JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Detection of Sialylated Phosphorylated κ-Casein Glycomacropeptide Electrophoresed on Polyacrylamide Gels and Cellulose Acetate Strips by the Thiobarbituric Acid and Malachite Green Dye Reactions

TAKUO NAKANO,*,† NORIAKI IKAWA,‡ AND LECH OZIMEK†

Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Alberta, Canada T6G 2P5, and Department of Agricultural Chemistry, Faculty of Agriculture, Meiji University, Kawasaki, Kanagawa 214-8571, Japan

A 64 amino acid residue sialylated phosphorylated glycomacropeptide (GMP) from bovine sweet whey can be detected as a Coomassie blue-staining peptide by electrophoresis on sodium dodecyl sulfate (SDS)−polyacrylamide gels. There is, however, limited information available concerning detection of GMP as a sialylated phosphorylated compound. Samples of GMP were electrophoresed on SDS−polyacrylamide gels or cellulose acetate strips (CAS). Immediately following electrophoresis, fractions obtained by cutting gels or strips were subjected to sialic acid determination by the thiobarbituric acid reaction and phosphorus determination by the malachite green dye reaction. Both determinations were found to be sensitive enough to detect approximately 20 and 40 μg of GMP in CAS and SDS gels, respectively. Further studies demonstrated that sialylated phosphorylated GMP can be detected on either SDS gels or CAS loaded with whey products or whey-added margarine residues.

KEYWORDS: Caseinomacropeptide; κ-casein glycomacropeptide; electrophoresis; sialic acid; whey; phosphorus; margarine

INTRODUCTION

Bovine sweet whey contains 64 amino acid residue sialylated phosphorylated glycomacropeptides (GMPs), which are released from κ-casein by the action of chymosin, a proteinase used to coagulate the milk casein complex during cheesemaking (see refs *1*−*4* for reviews). The proteinase cleaves the peptide bond between Phe-105 and Met-106 to yield insoluble para-κ-casein (residues 1−105), which remains with the curd formed, and soluble GMP (residues 106−169), which is released into sweet whey. GMP contains oligosaccharides including those composed of *N*-acetylneuraminic acid (sialic acid), galactose, and *N*-acetylgalactosamine, which are O-glycosidically linked to the peptide through threonine residues (e.g., Thr-131, Thr-133, and Thr-135; ref *1*). Sialic acid is a specific indicator of GMP accounting for approximately 80% of total sialic acid in sweet whey (*5*). Phosphorylation in GMP occurs at Ser-149 (*6*). GMP lacks aromatic amino acids (phenylalanine, tyrosine, and tryptophan) and is thought to be suitable for the source of dietary amino acids for patients suffering from phenylketonuria. GMP is also known to have various biological activities (e.g., protection against toxins, bacteria, and viruses and modulation of immune responses) (*2*−*4*). GMP as a dietary source of sialic acid may be important in brain growth of infants (*7*).

GMP has been isolated from sweet whey or casein hydrolysate and analyzed by various methods. Electrophoresis is one of the important techniques used to characterize GMP. On electrophoresis with sodium dodecyl sulfate−polyacrylamide gels (SDS gels), GMP gives a band stained with Coomassie blue R250 (CB), which is a dye commonly used to stain the peptide portion of GMP (*8*). However, not much is known about an electrophoretic method to detect GMP as a sialylated phosphorylated peptide. Such a method is needed for the characterization of GMP.

Glossmann and Neville (*9*) extracted glycoproteins from rat cell membranes, electrophoresed them on SDS gels, and detected sialic acid in gel slices by a colorimetric method with the thiobarbituric acid reaction. There is little information available concerning sialic acid assay in GMP electrophoresed on SDS gels. This study was undertaken to develop a method to determine phosphorus as well as sialic acid in GMP electrophoresed on SDS gels and cellulose acetate strips (CAS). Techniques developed using purified samples of GMP were then tested for the detection of GMP in whey products and whey-added margarine samples.

MATERIALS AND METHODS

Materials. Fresh bovine milk was obtained from the Dairy Unit at the University of Alberta Farm. Sweet whey was prepared from the fresh milk by chymosin treatment (*10*). It was dialyzed in water and freeze-dried to obtain whey protein concentrate (WPC). For preparation

* Author to whom correspondence should be addressed [telephone (780) 492-0381; fax (780) 492-4265; e-mail takuo.nakano@afhe.ualberta.ca].
† University of Alberta.
‡ Meiji University.

10.1021/jf062987f CCC: $37.00
Published on Web 03/10/2007

of acid whey, the fresh milk was adjusted to pH 4.6 with 1 N HCl and incubated at 50 °C for 20 min. The acid whey formed was then separated from casein and fat by centrifugation, dialyzed in water, and freeze-dried to obtain WPC. This product prepared without chymosin proteolysis contained no GMP when examined by gel chromatography on Sephacryl S-200, which showed no sialic acid peak with K_{av} at or near 0.40 seen in highly purified GMP (*5*). Commercial samples of WPC, whey protein isolate (WPI), and whey permeates were obtained from local milk processors. Samples of whey permeate were dialyzed in water, and the nondialyzable fractions obtained were used for chemical analysis and electrophoresis.

Diethylaminoethyl (DEAE)-Sephacel was a product of Pharmacia-Biotech, Baie d'Urfé, Quebec, Canada. Caseinoglycopeptide from bovine casein (GMPa), sialic acid (*N*-acetylneuraminic acid from sheep submaxillary mucin), and Sephacryl S-200-HR were obtained from Sigma-Aldrich Canada Ltd., Mississauga, ON, Canada. Bovine GMP (GMPb) was provided by Davisco Foods International, Inc., Eden Prairie, MN. Cellulose acetate strips (Sepraphore III, 2.5 cm × 15.2 cm) were obtained from Gelman Sciences Inc., Ann Arbor, MI. Malachite green was from Difco Laboratories, Detroit, MI. Margarine samples (each labeled as a whey-added product from a different processor) and those without whey were obtained from a local food store. These samples were delipidated with chloroform/methanol (2:1) (*11*). The insoluble residue obtained after delipidation was dialyzed in water to give a margarine residue.

Chromatographic Separation of GMP. A sample of WPC prepared from fresh milk (see Materials) was chromatographed on a DEAE-Sephacel anion-exchange column at pH 3.0 (*12*). Eluates obtained were monitored for sialic acid, a constituent sugar of GMP, and those containing GMP adsorbed on the anion-exchanger and eluted with 2 M NaCl were pooled, dialyzed in water, and freeze-dried. This preparation was further fractionated by gel chromatography on Sephacryl S-200-HR to obtain high-purity GMP.

Analytical Methods. The sialic acid content was determined by the thiobarbituric acid reaction (*5*) after hydrolysis of samples in 0.1 N sulfulic acid at 80 °C for 1 h. The reaction mixture for the sample of GMP preparation, whey product, or margarine residue consisted of 0.2 mL of hydrolysate of sample in 0.1 N sulfuric acid; 0.1 mL of 0.2 M sodium *m*-periodate/9 M phosphoric acid (solution A); 1 mL of 10% (w/v) sodium arsenite/0.5 M sodium sulfate/0.1 N sulfuric acid (solution B); and 3 mL of 0.6% (w/v) thiobarbituric acid/0.5 M sodium sulfate (solution C). The hydrolysate of sample was first incubated with solution A for 20 min at room temperature. Solution B was then added to the mixture of hydrolysate and solution A and mixed until a yellow-brown color disappeared. Solution C was added to this preparation, and the mixture was heated in boiling water for 15 min. The chromophore formed was extracted with 4.3 mL of 1-propanol, and its absorbance at 549 nm was read against blank solution prepared with 0.2 mL of 0.1 N sulfuric acid.

For the assay of sialic acid in SDS gel or CAS fractions (see below), the volume of reaction mixture was reduced to increase the sensitivity of the assay method. To a sample hydrolyzed in 0.2 mL of 0.1 N sulfuric acid was added a half-volume of each solution (0.05, 0.5, and 1.5 mL of solutions A, B, and C, respectively). The chromophore formed after heating the reaction mixture was extracted with 2.3 mL of 1-propanol, and its absorbance was read against blank solution prepared with SDS gel or CAS having no loaded sample. Absorbance was approximately 1.7-fold higher in the assay with reduced volumes of reaction mixture and solvent (1-propanol) compared to the original assay (see above).

The phosphorus content was determined by the malachite green dye reaction (*13*) in samples ashed at 550 °C overnight. Amino acid analysis was performed by using a Beckman model 6300 amino acid analyzer on samples hydrolyzed under nitrogen at 110 °C for 20 h in glass-distilled 6 N HCl containing 0.1% (w/v) phenol. The content of galactose was estimated by the anthrone reaction (*14*) using galactose as a standard, and that of galactosamine was estimated by the indole reaction (*15*) using galactosamine−HCl as a standard.

Electrophoresis of GMP on SDS Gels. Electrophoresis in 0.1% SDS was carried out on 12% polacrylamide gels in 0.1 M Tris−borate buffer, pH 8.6 (*16*). Gels were prepared using a mini-PROTEAN II cell (Bio-Rad, Mississauga, ON, Canada) with a 0.75 mm thick spacer and 10 well combs. Samples were dissolved in 0.04 M Tris−borate containing 2% (w/v) SDS and 8 M urea, pH 8.6, and boiled for 10 min prior to application. Electrophoresis for each sample was repeated until a sufficient number of gels were obtained for GMP staining and colorimetric determinations of sialic acid and phosphorus.

Gels were stained with 0.5% (w/v) CB in 50% (v/v) methanol containing 12.5% (w/v) trichloroacetic acid and destained in 5% (w/v) trichloroacetic acid (*8*). For colorimetric determinations of sialic acid and phosphorus, two adjoining wells were used for the same sample—one for sialic acid assay and the other for phosphorus assay. A portion of unstained gel, which was a strip (6 cm × 1 cm) containing a single lane (6 cm × 0.5 cm) in its inner part, was equally cut with a razor blade into 12 serial fractions of 0.5 cm × 1 cm in size. Fraction 1 contained the top of the gel corresponding to the origin, and fraction 12 contained the anodal end of the gel corresponding to the mobility of marker dye, bromophenol blue. Every fraction for sialic acid analysis was collected in a glass test tube containing 0.2 mL of 0.1 N sulfuric acid, cut into small pieces with the tip of spatula, and heated at 80 °C for 1 h to release sialic acid. More than 50% of the total sialic acid electrophoresed was released from the gel into 0.1 N sulfuric acid. Fractions used for phosphorus analysis were ashed at 550 °C overnight and subjected to the colorimetric assay with malachite green dye reaction (see above).

For sialic acid assay, unstained gels should be used. No significant amount of sialic acid was detected from CB-stained gels. Relatively large amounts of dye in the CB-stained gel also interfered with the assay by giving a blue color in the reaction mixture.

Electrophoresis of GMP on CAS. Samples were electrophoresed by applying a constant current of 1 mA/cm CAS for 1 h in either 0.1 M potassium phosphate buffer, pH 7.0, or 0.1 M pyridine/1.2 M acetic acid buffer, pH 3.5. Two samples were applied to a 2.5 cm wide strip giving a 1.25 cm wide space per sample. Following electrophoresis, the strip was stained with 0.5% (w/v) CB in 50% (v/v) methanol containing 12.5% trichloroacetic acid and destained with water. CAS was also subjected to a colorimetric determination of sialic acid or phosphorus. A portion (1.25 cm × 6 cm mini-strip) of each CAS loaded with GMP was equally cut to obtain 12 serial fractions of 1.25 × 0.5 cm in size, in that fraction 1 contained the origin and fraction 12 was the farthest portion of the mini-strip examined. For sialic acid analysis, every CAS fraction was collected in a glass test tube containing 0.2 mL of 0.1 N sulfuric acid and hydrolyzed as described above. Most (close to 100%) of the GMP sialic acid electrophoresed was released into 0.1 N sulfuric acid during hydrolysis. Sialic acid was assayed in CAS from both potassium phosphate and pyridine/acetic acid buffers. Phosphorus was determined in CAS from the pyridine/acetic acid buffer only. Phosphorus was not analyzed in CAS from phosphate buffer, which interfered with the assay by giving high absorbance in the blank solution.

Detection of GMP in Whey Products and Margarine Residues. Samples of WPC, WPI, whey permeate, and margarine residues were electrophoresed on either SDS gels or CAS. For SDS−gel electrophoresis, a sample (~5 mg) was mixed with 100 μL of sample buffer and heated in boiling water for 10 min. A 10 μL aliquot of supernatant obtained after centrifugation of the mixture at 12000*g* for 5 min was then mixed with 2 μL of 1% (w/v) bromophenol blue and applied to the well. For electrophoresis on CAS, a sample (~5 mg) was mixed with 100 μL of 0.1 M pyridine/acetic acid, pH 3.5, and a 10 μL aliquot of supernatant obtained after centrifugation of the mixture as described above was applied to the CAS in the same buffer. Two samples were loaded in a strip. Immediately following electrophoresis, unstained gels or CAS were subjected to sialic acid and phosphorus determinations.

RESULTS

Chromatographic Separation of GMP. An average 9.4% of WPC applied to the column of DEAE-Sephacel was adsorbed on the anion exchanger and eluted with 2 M NaCl as a single sialic acid peak, which accounted for 77.8% of total recovered sialic acid (chromatogram not shown). Chromatography of the sialic acid peak fraction on Sephacryl S-200 gave a single peak with $K_{av} = 0.39$ (**Figure 1**), accounting for 98.5% of total sialic

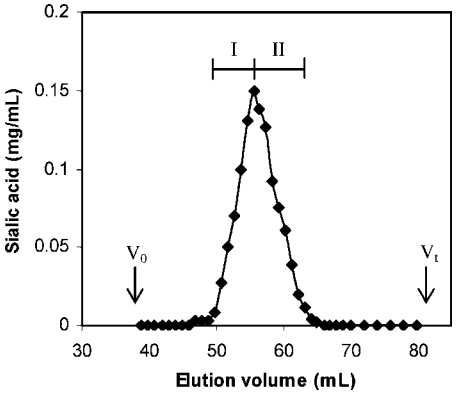

Figure 1. Chromatography of major sialic acid containing fraction from DEAE-Sephacel. A sample (67 mg) was dissolved in 1 mL of 0.1 M sodium acetate buffer, pH 7.0, and applied to a 1 × 110 cm column of Sephacryl S-200-HR equilibrated and eluted with the same buffer. Fractions of 1 mL were collected at a flow rate of 9 mL/h. Aliquots of 5 μL were removed and assayed for sialic acid. A bar indicates fractions pooled for further study. V_0 (void volume) and V_t (total column volume) were determined using blue dextran and tritiated water, respectively.

Table 1. Analysis of GMP Samples

	GMPa	GMPb	GMP I	GMP II
	Amino Acid (mol %)			
Asx	7.1	7.7	7.4	7.3
Thr	18.9	18.5	20.0	16.4
Ser	13.1	11.1	12.6	15.0
Glx	15.3	16.9	15.6	18.4
Gly	1.9	2.0	1.9	2.2
Ala	7.5	7.5	7.4	6.0
Val	7.8	7.3	7.6	7.2
Met	1.2	1.3	1.2	1.1
Ile	8.2	8.6	8.4	8.2
Leu	1.9	2.3	1.7	3.1
Tyr	nd[a]	nd	nd	nd
Phe	nd	0.3	nd	nd
His	0.2	0.2	nd	0.1
Lys	4.2	4.5	4.5	4.0
Arg	nd	0.3	nd	1.0
Pro	12.7	11.5	11.8	10.0
	Carbohydrate and Phosphorus (μg/mg of Dry Weight)			
sialic acid	65.0	77.6	116.7	94.4
galactose	47.1	44.2	71.5	50.2
galactosamine	27.8	47.5	64.2	56.9
phosphorus	7.0	5.8	4.6	6.3

[a] Not detected.

acid recovered. The K_{av} value was close to that (0.40) of GMP aggregates eluted from Sephacryl S-200 (*5*), indicating that the peak contained GMP sialic acid. The sialic acid containing eluates were pooled as indicated in **Figure 1** to obtain two fractions, I and II (referred to as GMP I and GMP II, respectively), which accounted for, respectively, 2.6 and 3.8% of WPC applied to the column of DEAE-Sephacel.

Table 1 shows chemical compositions determined in four GMP samples: GMPa; GMPb; GMP I; and GMP II. All samples except GMPb had undetectable levels of phenylalanine and tyrosine, which are the aromatic amino acids not found in GMP. GMPb had an undetectable amount of tyrosine and a negligible amount of phenylalanine. The sialic acid content was the highest in GMP I, higher in GMP II than in GMPb, and the lowest in GMPa. Variations were also observed in galactose, galactosamine, and phosphorus contents. However, the content of each carbohydrate or phosphorus was within the range of that of the corresponding GMP constituent reported elsewhere

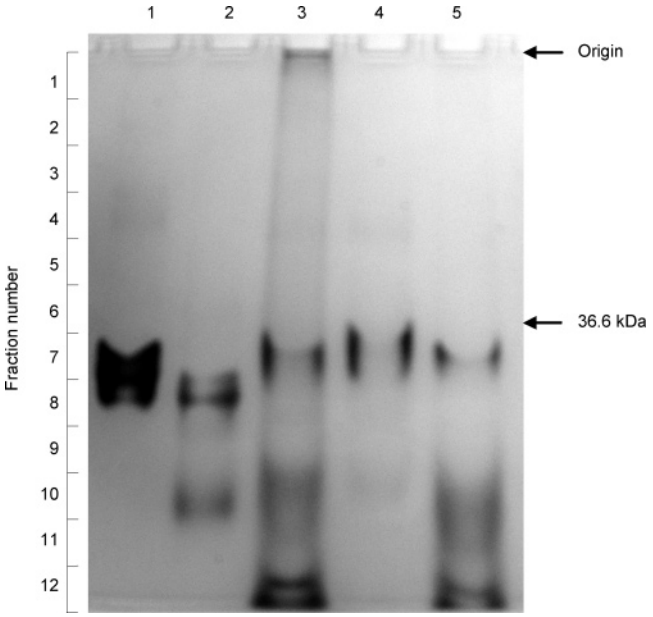

Figure 2. Electrophoresis of GMP preparations on SDS–polyacrylamide gels: lane 1, GMPa; lane 2, GMPb; lane 3, GMP-containing fraction from DEAE-Sephacel; lane 4, GMP I; lane 5, GMP II. Approximately 50 μg of sample was applied to the well. CB was used to stain GMP. The origin and the migration position of dimeric β-lactoglobulin (36.6 kDa) are shown. The vertical scale bar was given beside the gel so that one can compare the mobility of the CB-staining band with the positions of fractions, which were obtained by cutting an unstained gel for determination of sialic acid or phosphorus.

(*17*). These results indicated that all GMP preparations were of high purity usable for electrophoretic studies using SDS gels or CAS.

Electrophoresis of GMP on SDS Gels. All samples of bovine GMP showed a relatively broad CB-staining major band with average mobility greater than that of dimeric β-lactoglobulin (36.6 kDa) (**Figure 2**). This is consistent with the previously reported gel electrophoretic pattern of GMP (*18*) and provides evidence of the formation of aggregate of monomeric GMP with its molecular mass of ~8 kDa. The average mobility of GMP aggregate varied among the samples with R_f values being 0.60, 0.62, 0.55, and 0.57 for GMPa, GMPb, GMP I, and GMP II, respectively. A GMP fraction from DEAE-Sephacel, from which GMP I and GMP II were separated, had an R_f value of 0.56. The slightly lower mobility in GMP I than in GMP II confirms the chromatographic result (**Figure 1**). The higher contents of sialic acid, galactose, and galactosamine in GMP I than in GMP II (**Table 1**) may be related to the higher molecular size in GMP I. The CB-stained gel also showed minor band(s) in GMPa (R_f values of 0.29 and 0.32, both with weak staining), GMPb (R_f 0.84), GMP I (R_f values of 0.32 and 0.84, both with weak staining), and GMP II (R_f values 0.84 and 0.99).

Results of sialic acid and phosphorus determinations in gel fractions are shown in **Figure 3**. The peak of sialic acid content expressed as absorbance was seen at fraction 7 in GMPa and GMP I and at fraction 8 in GMPb and GMP II. The difference in the peak position between GMP I and GMP II is consistent with that in the mobility of CB-stained band between the two preparations of GMP (see above). The peak position apparently corresponded to the mobility of CB-stained band in GMPa, GMPb, and GMP I, but not in GMP II, which had its CB-staining band in the area corresponding to fraction 7 instead of fraction 8 (see above). The peak of phosphorus was seen at

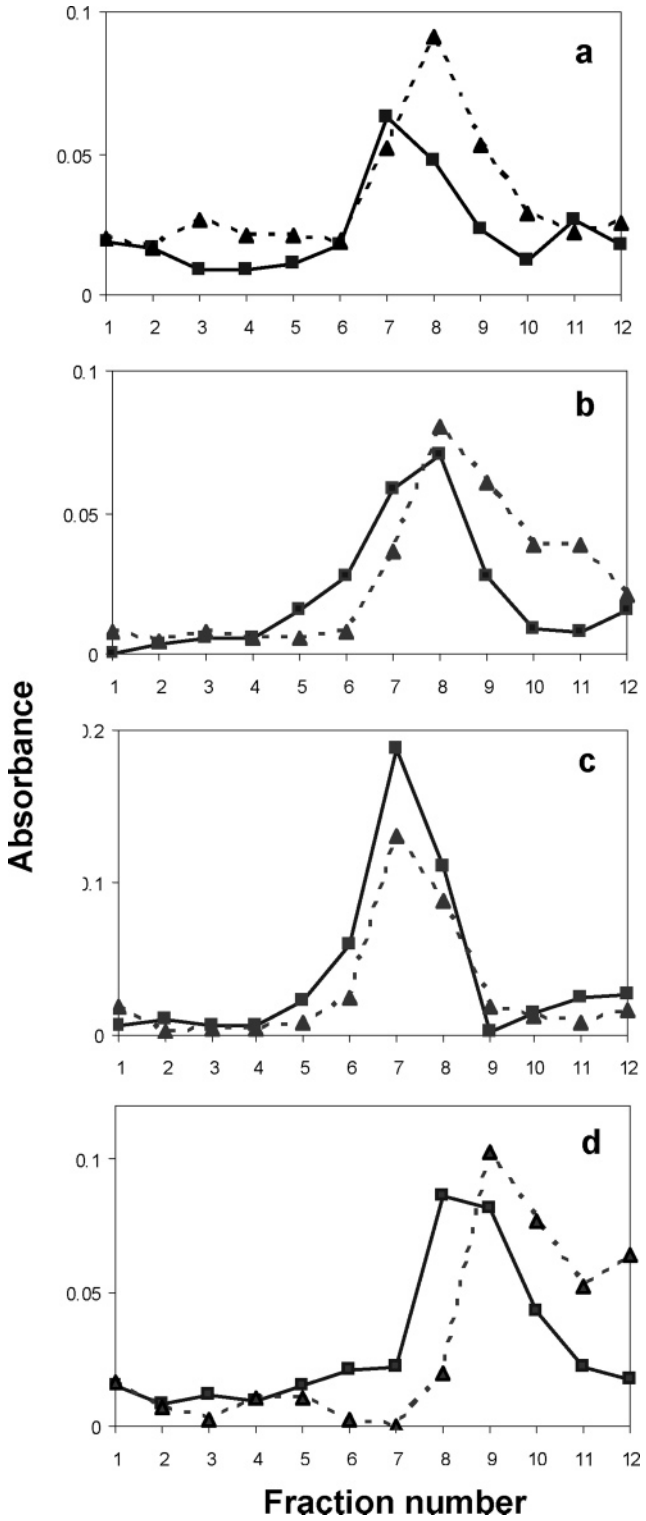

Figure 3. Analyses of sialic acid and phosphorus in SDS–polyacrylamide gels loaded with ~50 μg of GMP samples: (**a**) GMPa; (**b**) GMPb; (**c**) GMP I; (**d**) GMP II.Sialic acid was determined by the thiobarbituric acid reaction (absorbance at 549 nm, ■). Phosphorus was determined by the malachite green dye reaction (absorbance at 610 nm, ▲).

or near the position of the sialic acid peak in all samples examined, indicating that GMP is phosphorylated.

Detection of GMP in SDS Gels Loaded with Whey Products. In our preliminary experiment, SDS−gel electrophoresis of WPC gave three major CB-staining bands including those of monomeric and dimeric β-lactoglubulin (18.3 and 36.6 kDa, respectively) and α-lactalbumin (14.2 kDa). There was

Table 2. Sialic Acid and Phosphorus Concentrations (Micrograms per Milligram of Dry Weight) in Whey Products

sample[a]	sialic acid	phosphorus
	WPC	
1	13.4	3.2
2	16.3	2.0
3	12.8	1.4
4	14.6	2.7
5	13.0	2.8
6	14.3	1.5
7	24.3	4.1
8	6.1	2.0
	WPI	
1	13.2	1.5
2	12.7	1.0
3	1.1	0.1
	Whey Permeate	
1	0.1	21.6
2	0.1	141.0

[a] WPC sample 1 was prepared by us from fresh milk (see Materials). WPC samples 2–7 were commercial products. WPC sample 8 was prepared by us from acid whey. All samples of WPI and whey permeate were commercial products.

no clear band seen with the mobility of GMP (electrophoretogram not shown), which, if detectable, should show a band of GMP aggregate with mobility greater than that of dimeric β-lactoglobulin (see above). However, using the colorimetric methods we could detect sialic acid and phosphorus specific to GMP in most of the gels loaded with samples of WPC, WPI, and whey permeates (see **Table 2** for sialic acid and phosphorus concentrations determined in these samples prior to electrophoresis).

All WPC samples (**Figure 4**) except one (see below) had a sialic acid peak at fraction 7 or 8 with a phosphorus peak at or near the position of the sialic acid peak. This is consistent with the results obtained by electrophoresis of GMP (**Figure 3**). No appreciable peak of sialic acid was found in a sample of WPC from acid whey (containing no GMP, see Materials), as expected (**Figure 4h**). Therefore, significant amounts of phosphorus observed in fractions 1−8 from this sample were unlikely specific to GMP. Only one commercial sample (**Figure 4g**) had a sialic acid peak detected at fraction 3 with a relatively low content of phosphorus. No further investigation was carried out for this sialic acid containing material.

In WPI, a peak of sialic acid or phosphorus was seen in fraction 7 in two samples (**Figure 5a**,**b**) as found in WPC, whereas no peak was seen in a sample (**Figure 5c**) with a low content of sialic acid or phosphorus (**Table 2**). Electrophoresis of the nondialyzable fraction of whey permeates, accounting for an average of 1.2% of the dry weight of whey permeate, demonstrated that the first sample had two sialic acid peaks—one at fraction 5 and the other at fraction 9 (**Figure 6a**). The second sample gave a single sialic acid peak at fraction 7 (**Figure 6b**). All sialic acid containing fractions contained significant amounts of phosphorus.

Electrophoresis of GMP on CAS. Electrophoretic profiles with CB staining were different between samples or systems with different buffers. With 0.1 M potassium phosphate, pH 7.0 (**Figure 7**), GMPa (lane a) gave two broad bands with different staining intensities, whereas GMPb (lane b) gave three broad bands, among which the band with the slowest mobility was very weakly stained. The staining intensity of the fastest migrating band in GMPb was higher than that of the fast-migrating band of GMPa. Both GMP I (lane c) and GMP II (lane d) gave apparently single broad bands with higher staining

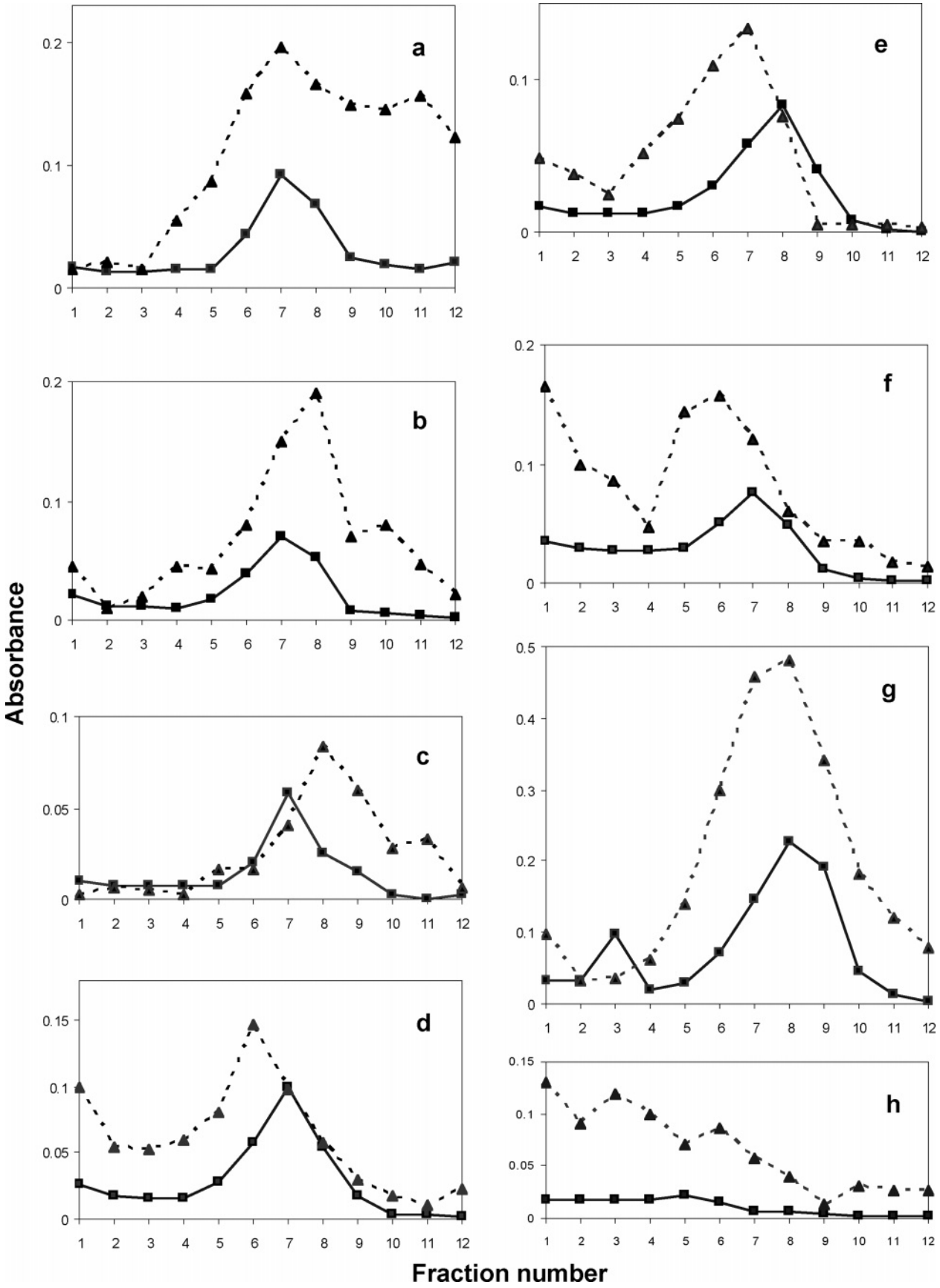

Figure 4. Analyses of sialic acid and phosphorus in SDS–polyacrylamide gels loaded with WPC: (■) sialic acid determined by the thiobarbituric acid reaction (absorbance at 549 nm); (▲) phosphorus determined by the malachite green dye reaction (absorbance at 610 nm); (**a**) sample prepared by us from fresh milk (see Materials); (**b**–**g**) commercial samples; (**h**) sample prepared from acid whey. Results shown in diagrams **a**–**h** were obtained with WPC samples 1–8 (**Table 2**), respectively.

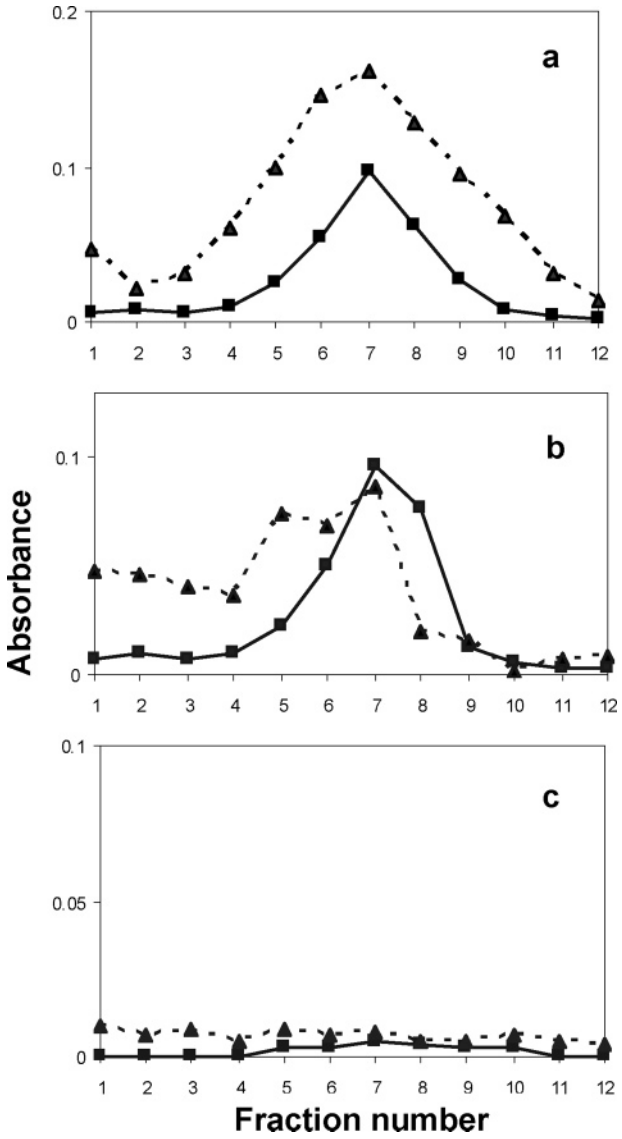

Figure 5. Analyses of sialic acid and phosphorus in SDS–polyacrylamide gels loaded with WPI: (■) sialic acid determined by the thiobarbituric acid reaction (absorbance at 549 nm); (▲) phosphorus determined by the malachite green dye reaction (absorbance at 610 nm). Results shown in diagrams **a**–**c** were obtained with WPI samples 1–3 (**Table 2**), respectively.

intensity in the latter. The mobilities of these bands were similar, but higher than that of the fast-migrating band from GMPa.

GMP samples electrophoresed on CAS in 0.1 M potassium phosphate buffer were then assayed for sialic acid (**Figure 8**). Phosphorus was not analyzed due to the difficulty of assay in the presence of potassium phosphate (see Materials and Methods). GMPa had a sialic acid peak at fraction 7, which corresponded to the area with little CB-stained band (**Figure 7**, lane a). No sialic acid was detected in fractions 5 and 6, which corresponded to the area showing a major CB-stained band from GMPa. The other samples (GMPb, GMP I, and GMP II) had a sialic acid peak at fraction 8. Therefore, in GMPb (**Figure 7**, lane b), only a small amount of sialic acid corresponding to ∼5% of total recovered sialic acid (estimated from absorbance values shown in **Figure 8b**) was detected in the areas corresponding to fractions 4−7 (**Figure 7**, lane b), where most of the CB-staining bands were seen. In contrast, most (∼70%, **Figure 8b**) of the sialic acid in GMPb was found in the area corresponding to the fast-migrating portion of the fastest

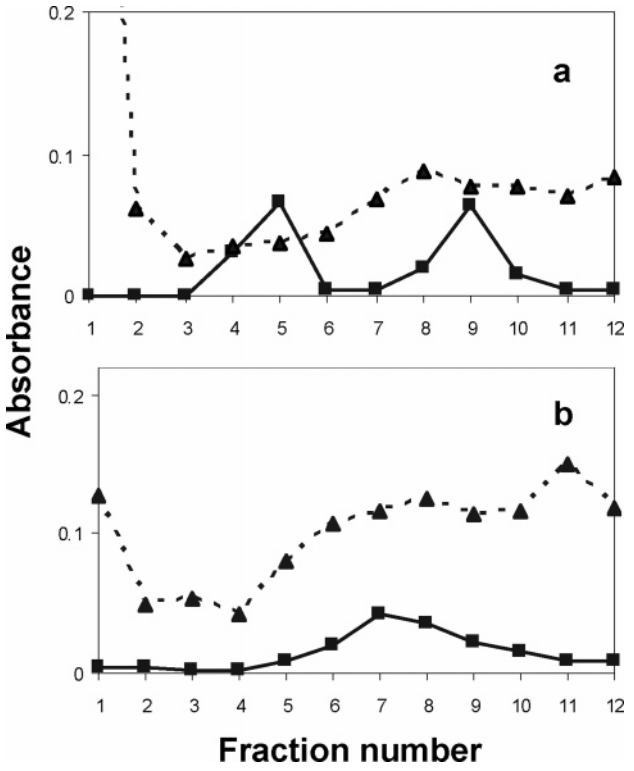

Figure 6. Analyses of sialic acid and phosphorus in SDS–polyacrylamide gels loaded with whey permeates: (■) sialic acid determined by the thiobarbituric acid reaction (absorbance at 549 nm); (▲) phosphorus determined by the malachite green dye reaction (absorbance at 610 nm). Results shown in diagrams **a** and **b** were obtained with whey permeate samples 1 and 2 (**Table 2**), respectively.

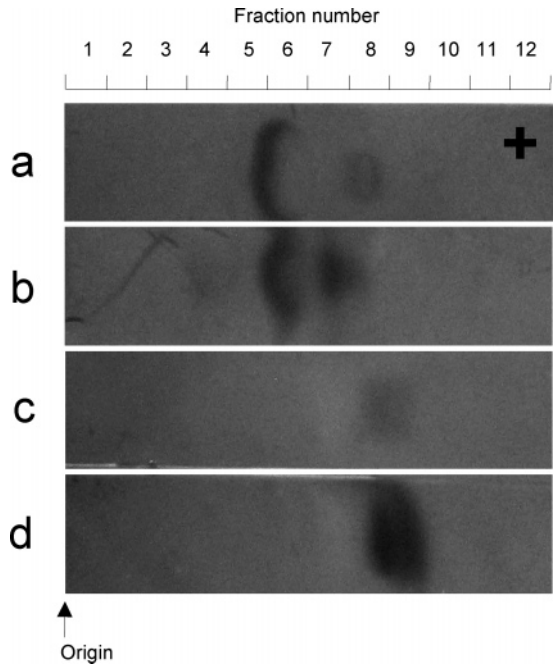

Figure 7. Electrophoresis of GMP preparations (∼50 μg) on CAS in 0.1 M potassium phosphate buffer, pH 7.0: (**a**) GMPa; (**b**) GMPb; (**c**) GMP I; (**d**) GMP II. The horizontal scale bar was given above the CAS so that one can compare the mobility of the CB-staining band with the positions of fractions, which were obtained by cutting an unstained CAS for determination of sialic acid or phosphorus.

CB-staining band. In GMP I and GMP II (**Figure 7**, lanes c and d, respectively), >60% of total recovered sialic acid (**Figure**

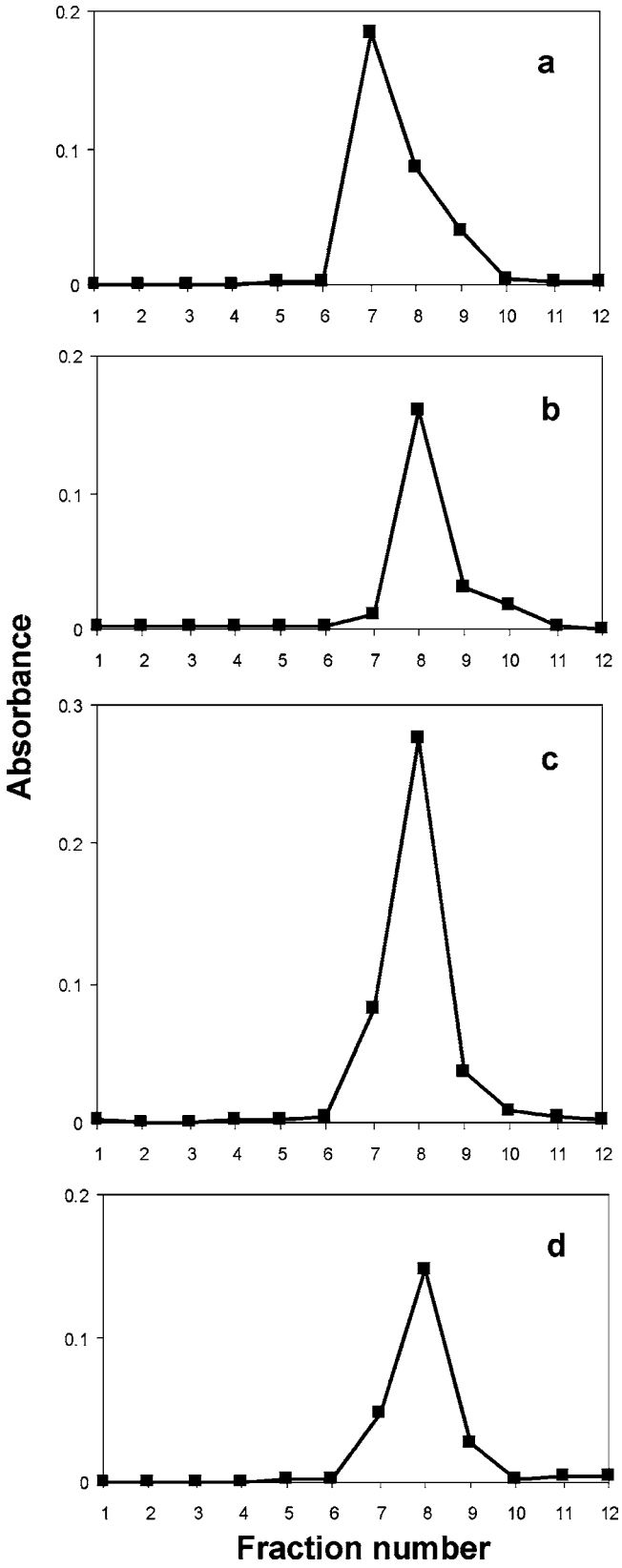

Figure 8. Analyses of of sialic acid in CAS loaded with GMP (~50 μg) in 0.1 M potassium phosphate buffer, pH 7.0: (**a**) GMPa; (**b**) GMPb; (**c**) GMP I; (**d**) GMP II; (■) sialic acid determined by the thiobarbituric acid reaction (absorbance at 549 nm).

8c,**d**) was found in the area corresponding to the slow-moving portion of the CB-staining band.

With pyridine/acetic acid, pH 3.5 (**Figure 9**), GMPa (lane a) had two discrete bands with different staining intensities,

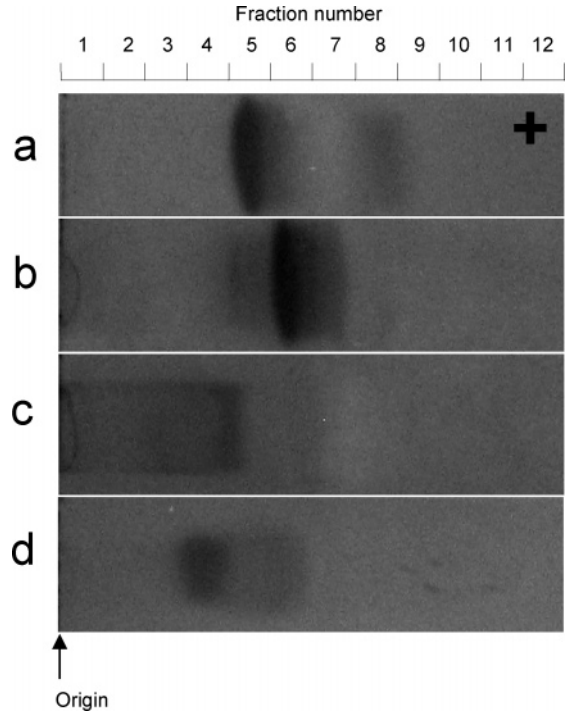

Figure 9. Electrophoresis of GMP preparations (~50 μg) on CAS in 0.1 M pyridine/acetic acid, pH 3.5: (**a**) GMPa; (**b**) GMPb; (**c**) GMP I; (**d**) GMP II. The horizontal scale bar was given above the CAS so that one can compare the mobility of the CB-staining band with the positions of fractions, which were obtained by cutting an unstained CAS for determination of sialic acid or phosphorus.

whereas GMPb (lane b) gave a very broad band the middle portion of which was densely stained. GMPb also gave a very weakly stained broad band with its mobility similar to that of the fast-migrating band of GMPa. GMP I (lane c) gave the broadest band extended from the origin to the position close to the end of the slow-moving band from GMPb. GMP I also had a very weakly stained broad band with its average mobility faster than that of the slow-migrating band of GMPb. GMP II (lane d) showed two bands—one had a mobility slower than the migrating front of major broad band from GMP I, and the other was a weakly stained band having its average mobility slower than that of the fast-migrating band from GMP I.

Sialic acid assay in CAS fractions (**Figure 10**) displayed a peak at fraction 6 in GMPa, at fraction 7 in GMP I and GMP II, and at fraction 8 in GMPb. The peak position for GMPa or GMPb apparently corresponded to the mobility of its weakly stained portion of the CB-staining band (**Figure 9**), whereas the peak position for GMP I or GMP II did not correspond to the mobility of its CB-stained band (**Figure 9**). The peak of phosphorus was seen at or near the position of the sialic acid peak in all samples examined. It was concluded that the sialic acid peak position is not closely related to the mobility of the CB-staining band on cellulose acetate electrophoresis in either potassium phosphate or pyridine/acetic acid buffer.

Detection of GMP in CAS Loaded with Whey Products. Results of sialic acid and phosphorus determinations in CAS loaded with WPC, WPI, and whey permeates are shown in **Figures 11**, **12**, and **13**, respectively. A sialic acid peak was found at fraction 7 or 8, as found in GMP samples (see above), in all samples of WPC and WPI analyzed, with the exception of those of WPC from acid whey (**Figure 11h**) and WPI with a low sialic acid content (**Figure 12c**), in which no sialic acid peak was seen, confirming gel electrophoresis results (see

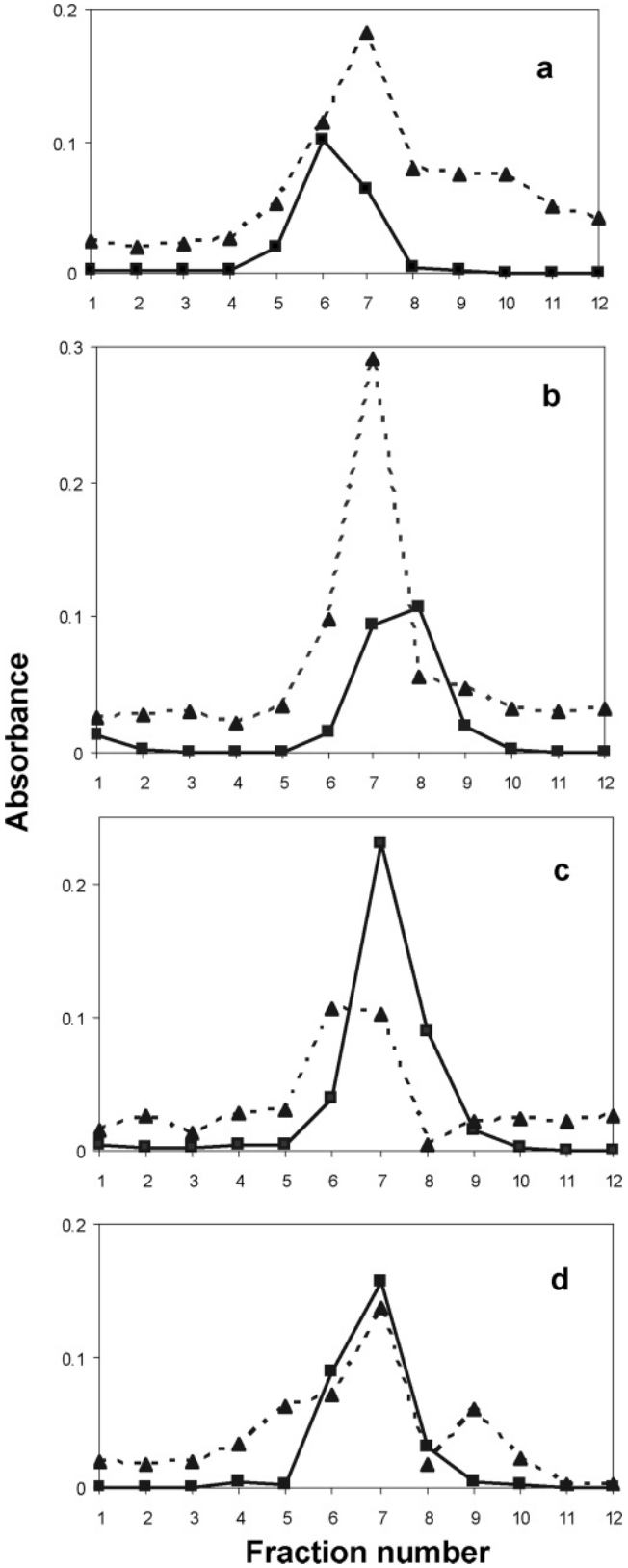

Figure 10. Analyses of sialic acid and phosphorus in CAS loaded with GMP (~50 μg) in 0.1 M pyridine/acetic acid, pH 3.5: (■) sialic acid determined by the thiobarbituric acid reaction (absorbance at 549 nm); (▲) phosphorus determined by the malachite green dye reaction (absorbance at 610 nm); (**a**) GMPa; (**b**) GMPb; (**c**) GMP I; (**d**) GMP II.

above). Sialic acid containing fractions showed significant amounts of phosphorus in all samples examined. Whey permeates gave sialic acid peaks at fractions 7 and 9 in sample 1 and in fractions 9 and 10 in sample 2 (**Figure 13b**) with significant amounts of phosphorus in all sialic acid containing fractions.

Detection of GMP in Whey-Added Margarine Residues. An average 0.08% (w/w) of whey-added margarine was extracted as margarine residues. Sialic acid and phosphorus concentrations determined in samples of margarine residues averaged 17.4 and 6.1 μg/mg of dry weight, respectively (**Table 3**). The portion of each sample was then electrophoresed on SDS gels or CAS, and fractions of gel or CAS obtained were monitored for sialic acid and phosphorus. All samples showed a sialic acid peak at fraction 7 or 8 with a significant amount of phosphorus on either electrophoresis (**Figure 14**). Margarine samples without whey, which had an undetectable level of sialic acid, were not studied.

DISCUSSION

CB has been used for the detection of GMP aggregates on SDS gels (*18*). However, this dye is not specific to carbohydrate moieties of GMP, and thus in the present study the electrophoretic mobility of sialylated GMP on SDS gels or CAS was determined by the assay of sialic acid, a specific indicator of GMP (*5*). Our results suggest that the mobility of the CB-staining band does not always reflect that of sialylated GMP on either SDS gel or CAS and that the sialic acid assay should be used to detect sialylated GMP. It appears that GMP with negatively charged sialic acid and phosphate has a low affinity to CB having a negative charge. The sialic acid assay, however, cannot be used for the detection of nonsialylated glycomacropeptide found as a relatively minor component in bovine sweet whey.

The thiobarbituric acid reaction used for sialic acid assay was a convenient, inexpensive, and reliable method, in that the chromophore that can be formed in the presence of gel or CAS fragments was extracted into a layer containing a water-miscible solvent, 1-propanol. The sensitivity of the assay was herein improved by reducing the volume of reaction mixture (see Analytical Methods). The sensitivity could further be improved by approximately 1.3 times by replacing 1-propanol with cyclohexanone (T. Nakano and L. Ozimek, unpublished observation), which is a water-immiscible solvent used to extract chromophore in the original method of thiobarbituric acid reaction reported by Warren (*19*). However, cyclohexanone was not used in this study because it is a highly hazardous solvent not practical for the assay of many samples. We have also tested other solvents including methanol, ethanol (95%), and 2-propanol, with which the absorbance of chromophore obtained was, respectively, 1.6, 1.4, and 1.4 times lower compared to that with 1-propanol.

Because the amount of sialic acid released during hydrolysis is greater in CAS than in SDS gels (see Materials and Methods), the latter requires a larger amount of sample (approximately twice) to be loaded for the detection of GMP sialic acid. Approximately 20 μg of GMP or 200 μg of whey products and margarine residues loaded on CAS can be easily detected by the sialic acid assay used in the present study. We have studied GMP using gel chromatography on Sephacryl S-200 and showed a GMP sialic acid peak eluting at or near the position of dimeric β-lactoglobulin (36.6 kDa) peak (*5*). The sample size and time required to detect sialylated GMP is much less in the electrophoretic methods developed in this study compared to the previous gel chromatographic method (*5*).

Little information is available concerning phosphorus determination in GMP electrophoresed on SDS gels or CAS. The malachite green dye reaction used in this study is a simple,

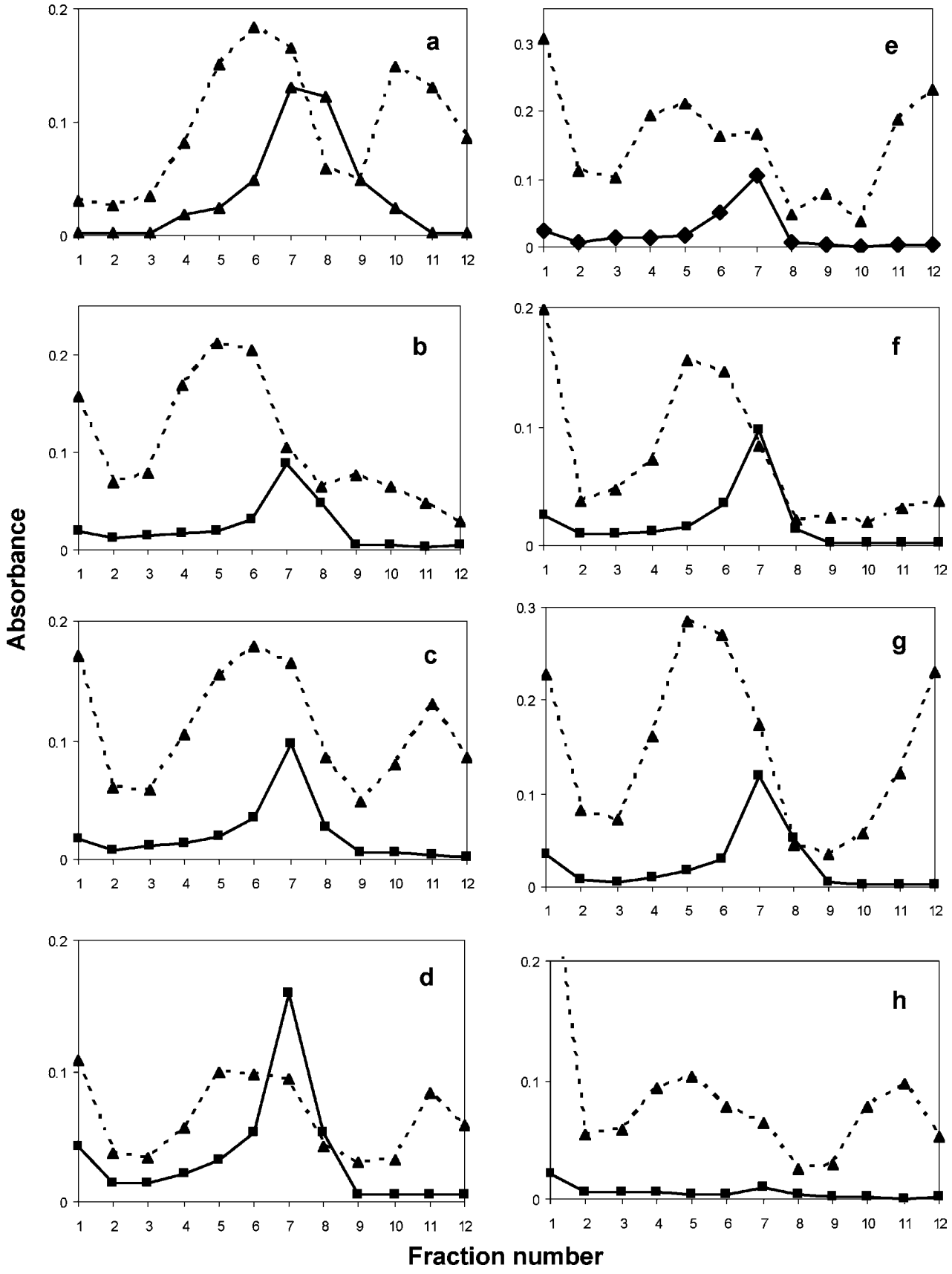

Figure 11. Analyses of sialic acid and phosphorus in CAS loaded with WPC: (■) sialic acid determined by the thiobarbituric acid reaction (absorbance at 549 nm); (▲) phosphorus determined by the malachite green dye reaction (absorbance at 610 nm); (**a**) sample prepared by us from fresh milk (see Materials); (**b**–**g**) commercial samples; (**h**) sample prepared by us from acid whey. Results shown in diagrams **a**–**h** were obtained with WPC samples 1–8 (**Table 2**), respectively.

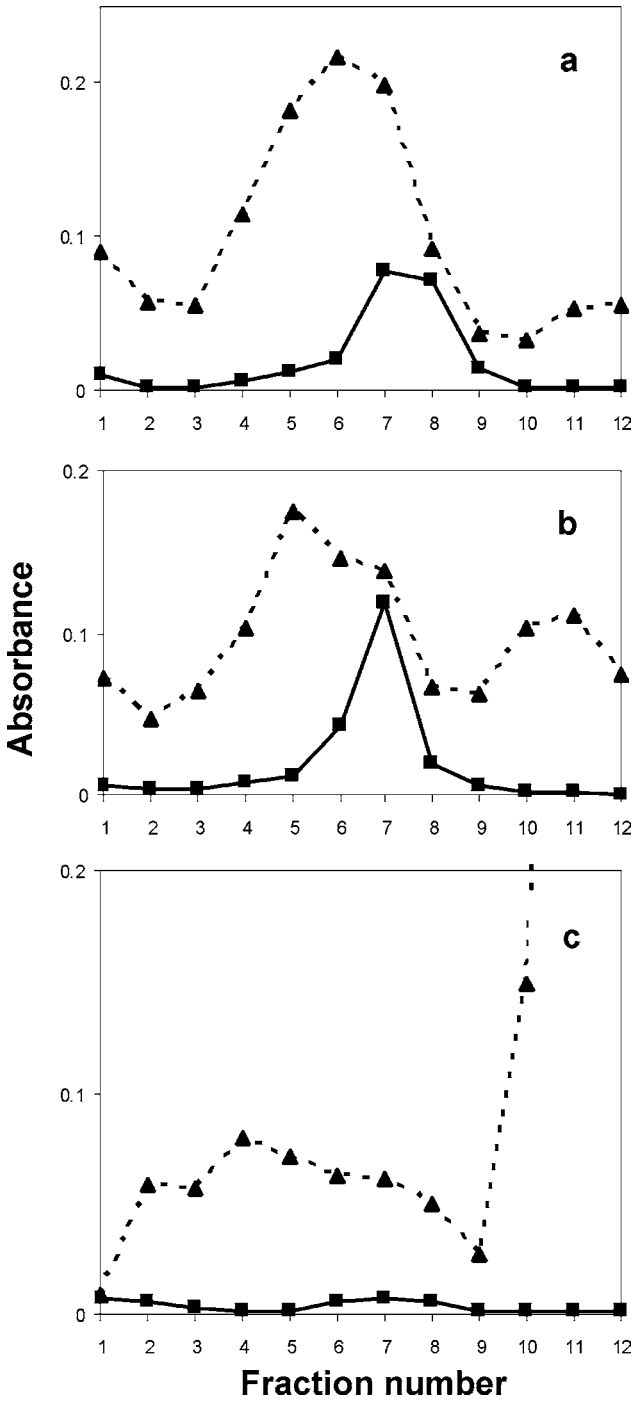

Figure 12. Analyses of sialic acid and phosphorus in CAS loaded with WPI: (■) sialic acid determined by the thiobarbituric acid reaction (absorbance at 549 nm); (▲) phosphorus determined by the malachite green dye reaction (absorbance at 610 nm). Results shown in diagrams **a**–**c** were obtained with WPI samples 1–3 (**Table 2**), respectively.

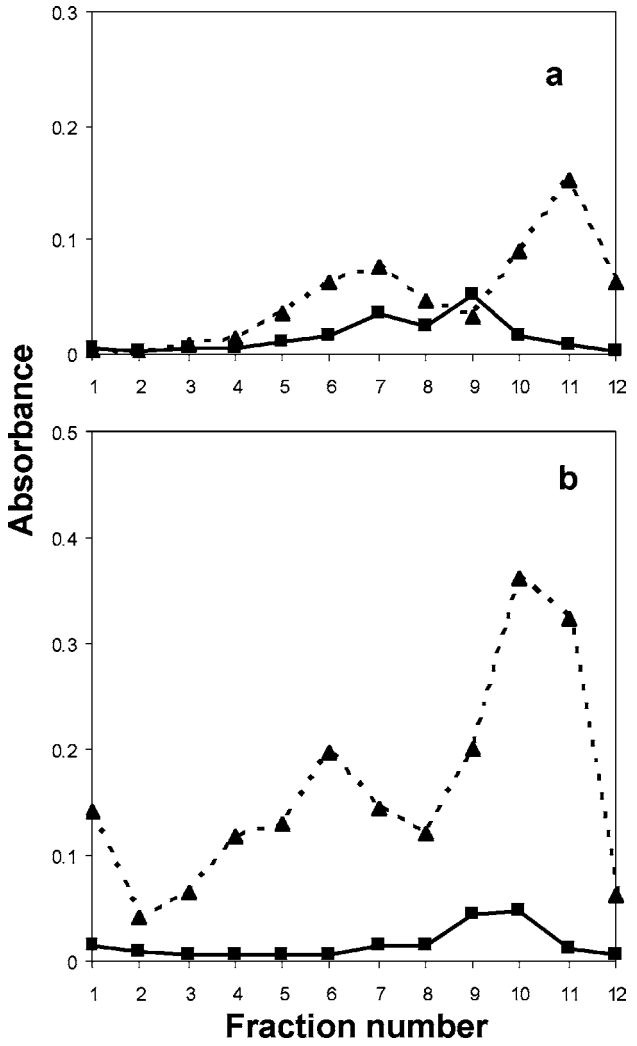

Figure 13. Analyses of sialic acid and phosphorus in CAS loaded with whey permeates: (■) sialic acid determined by the thiobarbituric acid reaction (absorbance at 549 nm); (▲) phosphorus determined by the malachite green dye reaction (absorbance at 610 nm). Results shown in diagrams **a** and **b** were obtained with whey permeate samples 1 and 2 (**Table 2**), respectively.

Table 3. Sialic Acid and Phosphorus Concentrations (Micrograms per Milligram of Dry Weight) in Whey-Added Margarine Residues

sample	sialic acid	phosphorus
1	20.7	3.0
2	20.9	4.3
3	12.8	3.3
4	18.6	9.4
5	13.8	10.5

inexpensive, and sensitive method. Our results now confirm that GMP is a phosphorylated peptide derived from bovine κ-casein (*1*−*4*, *6*).

We have tested fluorescent dyes, Pro-Q Emerald 488 and Pro-Q Diamond (both obtained from Invitrogen Corp., Burlington, ON, Canada), for staining GMP on SDS gels (T. Nakano and L. Ozimek, unpublished data). Pro-Q Emerald 488 reacts with periodate-oxidized carbohydrates to produce fluorescence on glycoproteins, whereas Pro-Q Diamond reacts with phosphate groups attached to tyrosine, serine, and threonine residues. Little information is available concerning staining of GMP with these fluorescent dyes. Using these dyes, we could visualize fluorescent bands of GMP samples and confirmed that they are glycosylated phosphorylated compounds. However, staining intensity varied among samples, and the mobility of the fluorescent band did not always reflect that of sialylated or phosphorylated GMP determined by the colorimetric analysis used in this study. Thus, we concluded that staining gels with these fluorescent dyes is not reliable for the detection of glycosylated phosphorylated GMP. We have also attempted to detect GMP on gels using periodic acid/Schiff staining (*9*) and confirmed that the GMP aggregate was localized as a major band with its mobility similar to that stained with CB (T. Nakano and L. Ozimek, unpublished data). However, the sensitivity of

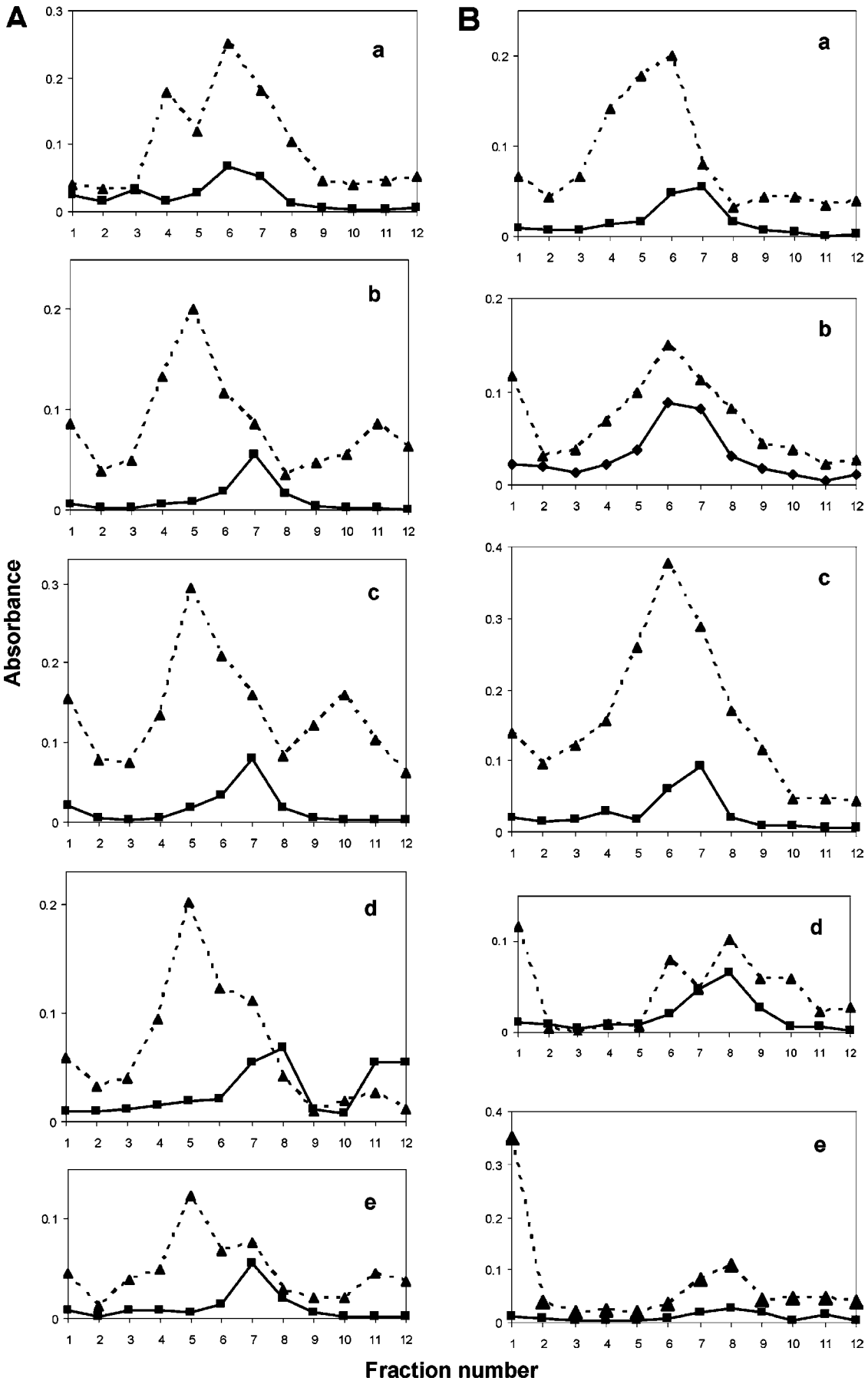

Figure 14. Analyses of sialic acid and phosphorus in SDS–polyacrylamide gels (**A**) and CAS (**B**) both loaded with margarine residues: (■) sialic acid determined by the thiobarbituric acid reaction (absorbance at 549 nm); (▲) phosphorus determined by the malachite green dye reaction (absorbance at 610 nm). Results shown in diagrams **a**–**e** were obtained with margarine residue samples 1–5 (**Table 3**), respectively.

this method was approximately 10 times lower than that with the sialic acid assay used in the present study.

Cellulose acetate electrophoresis of acid whey proteins has been reported (*20*). However, there is little information available concerning cellulose acetate electrophoresis of GMP. We have tested electrophoresis of GMP using a procedure similar to that reported by the previous authors (*20*) and found that Ponceau S, an anionic dye used by them to stain whey proteins separated on CAS, does not stain GMP on CAS (T. Nakano and L. Ozimek, unpublished observation). Our preliminary study also demonstrated that (1) GMP having an isoelectric point (p*I*) < 3.8 (*12*) migrates more rapidly than do most bovine whey proteins (p*I* > 4) including β-lactoglobulin, α-lactalbumin, IgG, and serum albumin on cellulose acetate electrophoresis in potassium phosphate buffer, pH 7.0; and (2) in pyridine/acetic acid buffer, pH 3.5, the above whey proteins do not show any significant positive mobilities, whereas GMP has a relatively fast positive mobility. Cellulose acetate electrophoresis may be a useful method for a small-scale isolation and purification of GMP from WPC, assuming that most of the GMP electrophoresed will be recovered from CAS.

It has been reported that WPI prepared by membrane technology with microfiltration and ultrafiltration contains GMP, which is not found in WPI prepared with an ion-exchange process (*21*). The WPI with very low concentrations of GMP sialic acid and phosphorus found in this study (**Figures 5c** and **12c**) might have been prepared with an ion-exchange process.

There is little information available concerning analysis of whey permeate. The extremely high content of phosphorus found in sample 2 (**Table 3**) may be due to the presence of phosphorus-rich insoluble matter present in it. Results of SDS gel electrophoresis suggest that the sialic acid peak at fraction 9 from sample 1 (**Figure 6a**) and that at fraction 7 from sample 2 (**Figure 6b**) may contain degraded product of GMP and nondegraded aggregated GMP, respectively. Electrophoretic profiles on CAS were also different between the two samples (**Figure 13**). It is unknown whether the two sialic acid peaks observed on CAS are related to those observed on SDS gels.

Sweet whey has been used as an ingredient in various processed foods (e.g., margarine, bakery products, and beverages). In commercial margarine, sweet whey is used as an emulsifier to stabilize the water-in-oil emulsion. The present study clearly showed that the whey-added margarine samples contain sialylated phosphorylated bovine GMP (and thus sweet whey, but not acid whey). Interestingly the sialic acid concentrations in margarine residues (13−21 μg/mg of dry weight, **Table 3**) are fairly comparable to those in WPC and WPI (13−24 μg/mg of dry weight, **Table 2**).

The present study demonstrates an inexpensive and reliable qualitative method, which can be used for the detection of GMP in sweet whey-added food products including margarine. It is suggested that sialic acid assay by the thiobarbituric acid reaction is the first step, and then electrophoresis on SDS gels or CAS is carried out to detect sialylated phosphorylated GMP by sialic acid and phosphorus determinations. Cellulose acetate electrophoresis, which requires less time than SDS−gel electrophoresis, may be more convenient, but SDS−gel electrophoresis is also an important technique to confirm the formation of GMP aggregates, a property characteristic to this peptide. The high recovery (~100%, see Materials and Methods) of GMP sialic acid from CAS observed in this study suggests that cellulose acetate electrophoresis may be used for the quantitative determination of GMP sialic acid. It appears that the sialic acid and phosphorus analyses described here can be applied to electrophoretic characterization of sialylated and/or phosphorylated proteins (or peptides other than GMP).

In conclusion, this study provides previously unreported information on the method of detection of sialylated phosphorylated GMP on SDS gels and CAS by analyzing sialic acid and phosphorus with the thiobarbituric acid and malachite green dye reactions, respectively. The method is specific and can be directly applied to the detection of GMP in whey products and whey-added margarine.

ABBREVIATIONS USED

CAS, cellulose acetate strips; CB, Coomassie blue R250; DEAE, diethylaminoethyl; GMP, glycomacropeptide; K_{av}, partition coefficient; p*I*, isoelectric point; SDS, sodium dodecyl sulfate; WPC, whey protein concentrate; WPI, whey protein isolate.

LITERATURE CITED

(1) Eigel, W. N.; Butler, J. E.; Ernstrom, C. A.; Farrel, H. M., Jr.; Harwalker, V. R.; Jeness, R.; Whitney, R. McL. Nomenclature of proteins of cow's milk: fifth revision. *J. Dairy Sci.* **1984**, *67*, 1599−1631.

(2) Abd El-Salam, H. M.; El-Shibiny, S.; Buchheim, W. Characteristics and potential uses of the casein macropeptide. *Int. Dairy J.* **1996**, *6*, 327−341.

(3) 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.

(4) Brody, E. P. Biological activities of bovine glycomacropeptide. *Br. J. Nutr.* **2000**, *84* (Suppl. 1), S39−S46.

(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) Rasmussen, L. K.; Sorensen, E. S.; 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.

(7) Nakano, T.; Sugawara, M.; Kawakami, H. Sialic acid in human milk: composition and function. *Acta Paediatr. Tw.* **2001**, *42*, 11−17.

(8) 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.* **1998**, *7*, 559−569.

(9) Glossmann, H.; Neville, D. M., Jr. Glycoproteins of cell surfaces. A comparative study of three different cell surfaces of the rat. *J. Biol. Chem.* **1971**, *246*, 6339−6346.

(10) Chu, L.; MaCleod, A.; Ozimek, L. Isolation of glycomacropeptide from sodium caseinate hydrolysate solution by ultrafiltration. *Milchwissenschaft* **1996**, *51*, 303−306.

(11) Folch, J.; Lees, M.; Sloan Stanley, G. K. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **1957**, *226*, 497−507.

(12) Nakano, T.; Ozimek, L. Purification of glycomacropeptide from dialyzed and non-dialyzed sweet whey by anion-exchange chromatography at different pH values. *Biotechnol. Lett.* **2000**, *22*, 1081−1086.

(13) Van Veldhoven, P. P.; Mannaerts, G. P. Inorganic and organic phosphate measurements in the nanomolar range. *Anal. Biochem.* **1987**, *161*, 45−48.

(14) Trevelyan, W.; Harrison, J. S. Studies of yeast metabolism. I. Fractionation and microdetermination of cell carbohydrates. *Biochem. J.* **1952**, *50*, 298−303.

(15) Dische, Z.; Borenfrend, E. A spectrophotometric method for the micro-determination of hexosamines. *J. Biol. Chem.* **1950**, *184*, 517−522.
(16) Mechanic, G. L. Collagen biochemistry. In *Skeletal Research*; Simmons, D. J., Kunin, A. S., Eds.; Academic Press: New York, 1979; pp 227−241.
(17) 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.
(18) Nakano, T.; Silva-Hernandez, E. R.; Ikawa, N.; Ozimek, L. Purification of κ-casein glycomacropeptide from sweet whey with undetectable level of phenylalanine. *Biotechnol. Prog.* **2002**, *18*, 409−412.
(19) Warren, L. The thiobarbituric acid assay of sialic acids. *J. Biol. Chem.* **1959**, *234*, 1971−1975.
(20) Bell, J. W.; Stone, W. K. Rapid separation of whey proteins by cellulose acetate electrophoresis. *J. Dairy Sci.* **1979**, *62*, 502−504.
(21) Deshler, M. Whey protein isolates: Production, composition and nutrition facts. *Nutraceuticals World 1999*; online, available at http://www.nutraceuticalsworld.com/marapr99-b8.htm.

Received for review October 17, 2006. Revised manuscript received February 5, 2007. Accepted February 11, 2007. This study was supported by grants from Parmalat Canada and the Meiji University Science and Technology Research Institute.

JF062987F