Determination of Sialic Acid by the Thiobarbituric Acid Reaction in Sweet Whey and Its Fractions

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Determination of sialic acid in sweet whey is useful as the concentration of sialic acid reflects the amount of glycomacropeptide (GMP) present. In this study, the concentration of total sialic acid was determined by the thiobarbituric acid reaction after dialysis of samples in water, and the concentration of GMP sialic acid was estimated by gel chromatography on Sephacryl S-200. Concentrations of total and GMP sialic acid determined in a sweet whey sample prepared from fresh milk were 2.0 and 1.5 μg/mg of dry weight, respectively. Analysis of commercial samples showed that the concentration of total sialic acid in sweet whey was 9 times lower than that in whey protein concentrate but 18 times higher than that in whey permeate. A similar trend was observed in the variation of GMP sialic acid concentration between sweet whey and why protein concentrate. The concentration of sialic acid differed 10 times between two samples of whey protein isolate.

Keywords: *Sialic acid; cheese whey; glycomacropeptide*

INTRODUCTION

Sialic acid is a constituent sugar of κ-casein glycomacropeptide (GMP), which is a biologically active compound (Abd El-Salam et al., 1996; Dziuba and Minkiewicz, 1996) found in rennet whey (sweet whey). We have previously studied GMP by gel chromatrography and reported that the majority of sialic acid in a nondialyzable fraction of sweet whey was GMP sialic acid (Nakano and Ozimek, 1998). This suggests that the determination of sialic acid in sweet whey is important to estimate the concentration of GMP present. However, there are few reports of attempts to determine the sialic acid concentration in sweet whey. Sialic acid is commonly determined by the thiobarbituric acid reaction (Warren, 1959). This technique cannot be applied directly to the estimation of sialic acid concentration in sweet whey because sweet whey contains materials (e.g., lactose and riboflavin; T. Nakano and L. Ozimek, unpublished data) that interfere with the assay by producing anomalous chromophores. The objectives of this study were, therefore, (1) to examine three techniques including dialysis, gel chromatography, and ion-exchange chromatography to remove interfering substances from sweet whey and (2) to determine concentrations of total and GMP sialic acid in samples of sweet whey and its fractions.

MATERIALS AND METHODS

Materials. A sample of sweet whey was prepared from fresh milk by chymosin treatment (Chu et al., 1996) followed by centrifugation at 20000g and 4 °C for 30 min to remove fat and protein precipitate. The supernatant obtained was used for the experiment to examine the recovery of sialic acid from sweet whey after dialysis or chromatography (see below). A sample of GMP was prepared from sodium caseinate as described (Nakano and Ozimek, 1998). Commercial samples of sweet whey, whey permeate, whey protein concentrate, and whey protein isolate (Table 1) were also analyzed in this study. Suppliers' information showed that protein and lactose concentrations in sweet whey (samples I and II) averaged 11 and 71 g/100 g of dry weight, respectively. Corresponding values for whey protein concentrate (samples I−V) were 79 and 7 g/100 g of dry weight, and those for whey protein isolate (samples I and II) were 95 and <1 g/100 g of dry weight, respectively. Information of fat concentration was also available for sweet whey sample II (1 g/100 g of dry weight), whey protein concentrate samples I, III, IV, and V (average = 5 g/100 g of dry weight), and whey protein isolate samples I and II (<1 g/100 g of dry weight). There was no supplier's information available on the chemical composition of whey permeate. Every sample within a product type was from a different supplier.

Table 1. Sialic Acid Concentrations in Commercial Samples

sample	sialic acid (μg/mg of dry wt)	sample	sialic acid (μg/mg of dry wt)
sweet whey		WPC[a]	
I	2.0	I	18.0
II	1.6	II	16.6
whey permeate		III	14.9
I	0.1	IV	15.1
II	0.1	V	15.0
		WPI[b]	
		I	1.7
		II	17.3

[a] Whey protein concentrate. [b] Whey protein isolate.

Recoveries of Sialic Acid after Dialysis and Chromatography. A 1 mL aliquot of sweet whey in six replicates was transferred into dialysis tubes with a 6−8 kDa molecular weight (MW) cutoff and dialyzed in running tap water for 24 h and then for another 24 h in deionized water at 4 °C. The loss of sialic acid, if any, during dialysis was not checked because we intended to estimate the content of sialic acid from GMP, which is not dialyzable. The content of sialic acid retained in a dialysis tube was determined by the thiobarbituric acid reaction (Warren, 1959) after hydrolysis in 0.1 N sulfuric acid at 80 °C for 1 h. The chromophore formed in the reaction mixture was extracted using 1-propanol (Nakano et

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al., 1994) instead of cyclohexanone as used in the original method (Warren, 1959). *N*-Acetylneuraminic acid from sheep submaxillary glands (Sigma Chemical Co., Mississauga, ON, Canada) was used as a standard sialic acid.

Another aliquot (1 mL) of sweet whey in four replicates was separately applied to a gel chromatography column on Sephacryl S-200 (Pharmacia Biotech Inc., Baie d'Urfé, PQ, Canada) equilibrated and eluted with 0.1 M sodium acetate buffer, pH 7.0, containing 0.02% sodium azide. All fractions collected were measured for their volumes because protein or GMP fractions had lower volumes than did nonprotein fractions. Aliquots of fractions were then monitored for the contents of protein (by measuring absorbance at 280 nm) and of sialic acid. For the latter, 196 μL aliquots of fractions were mixed with 4 μL of 9 N sulfuric acid, to obtain pH ~1.4, and heated at 80 °C for 1 h. After heating, the sialic acid content in each aliquot was determined by the thiobarbituric acid reaction as described above. Fractions showing anomalous chromophores were pooled, dialyzed in water, and redetermined for sialic acid after concentration by freeze-drying. Blue dextran (Pharmacia Biotech Canada Inc.) and tritiated water were used to determine void volume (V_0) and total column volume (V_t), respectively. The partition coefficient (K_{av}) of the sialic acid peak was calculated from the formula $K_{av} = (V_e - V_0)/(V_t - V_0)$, in which V_e represents the volume of the peak fraction. No attempt was made in this study to estimate the MW of GMP because there were no appropriate glycoprotein or glycopeptide standards available to calibrate the column. Use of protein standards to calibrate a column results in an overestimation of the apparent MW of GMP (Nakano and Ozimek, 1998).

The third aliquot (1 mL) of sweet whey in four replicates was then separately applied to an ion-exchange column on DEAE-Sephacel (Pharmacia Biotech, Canada Inc.) equilibrated with 0.01 M sodium acetate buffer, pH 7.5, containing 0.02% sodium azide. The materials adsorbed in the column were eluted with the equilibration buffer containing 1 M NaCl. Aliquots of fractions collected were monitored for protein and sialic acid, and fractions showing anomalous chromophores were redetermined for sialic acid as described above for Sephacryl S-200 chromatography.

Analysis of Commercial Samples. Sialic acid concentrations were then determined in commercial samples of sweet whey, whey permeate, and whey protein isolate (Table 1). All samples of sweet whey and whey permeate and one sample of whey protein concentrate (sample I, Table 1) with relatively high concentration (24 g/100 g of dry weight) of lactose were dialyzed in water as described above to remove materials that interfere with the sialic acid assay. All other samples including those of four whey protein concentrates and two whey protein isolates (Table 1) were prepared as suspensions in water, and not dialyzed. Preliminary analysis showed that these samples had no anomalous chromophores in their reaction mixtures with thiobarbituric acid. A portion of every commercial sample in water was taken by stirring, hydrolyzed in 0.1 N sulfuric acid at 80 °C for 1 h, and assayed for sialic acid as described above. The other portion of each sample was centrifuged, and the supernatant obtained was chromatographed on Sephacryl S-200. Samples of sweet whey and whey permeate were concentrated by freeze-drying before application to the column.

Analytical data with greater than two replicates were calculated as the mean ± standard deviation (SD). A *t* test was used to detect significant differences between means.

RESULTS AND DISCUSSION

The content of total sialic acid recovered from sweet whey after dialysis was 116 ± 4 μg/mL, which corresponded to 1.9 ± 0.1 μg/mg of dry weight. (The sweet whey sample contained 6.1% dry matter.) This was higher with lower variations ($P < 0.05$) than the content of total sialic acid recovered from either the Sephacryl S-200 (106 ± 11 μg/mL) or DEAE-Sephacel (103 ± 14 μg/mL) column. Therefore, for a quantitative analysis of sialic acid, dialysis was considered to be more practical compared to chromatographic methods to remove interfering substances from sweet whey. However, Sephacryl S-200 chromatography appeared to be useful to separate a peak of GMP, and DEAE-Sephacel to concentrate sialic acid containing materials (see below). Sephacryl S-200 chromatography (Figure 1) gave three peaks (K_{av} = 0, 0.40, and 0.86) accounting for approximately 4, 87, and 9% of recovered sialic acid, respectively. The third peak having an anomalous chromophore had been apparently largest among the three (Figure 1) but was found to contain only a small proportion of recovered sialic acid after dialysis. The major peak (elution volumes - 46−60 mL), having K_{av} identical to that of the peak of GMP aggregate (K_{av} = 0.40, Figure 1) was, thus, identified to be a peak of GMP sialic acid. The elution position of GMP sialic acid was close to that (K_{av} = 0.39) of β-lactoglobulin (data not shown). The total content of GMP sialic acid calculated from the chromatogram (Figure 1) was 92 μg/mL or 1.5 μg/mg dry weight. On DEAE-Sephacel chromatography, most sialic acid (92 ± 2% of total) from sweet whey was found in fractions adsorbed in the column and eluted with 1 M NaCl (Figure 2). Gel chromatography of the adsorbed fraction on Sephacryl S-200 gave a GMP peak corresponding to 80% of recovered sialic acid (chromatogram not shown).

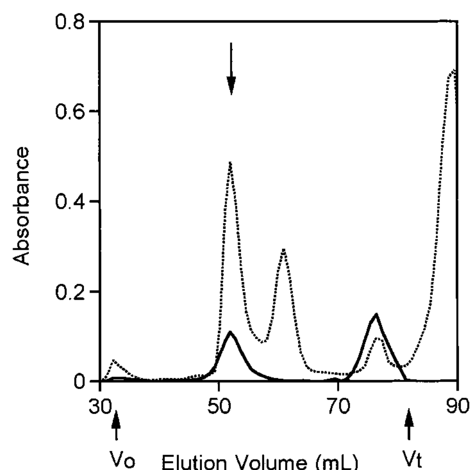

Figure 1. Representative Sephacryl S-200 chromatogram of sweet whey: (—) absorbance at 549 nm for sialic acid; (- - -) absorbance at 280 nm for protein. A portion of sweet whey was applied to a 1 × 109 cm column of Sephacryl S-200, and fractions were collected at a flow rate of ~9 mL/h at 21 °C. The arrow (at the elution volume of 52 mL) shows the position of the GMP sialic acid peak. Fractions with elution volumes of 73−82 mL showed anomalous chromophores. V_0 and V_t show the void volume and total column volume, respectively. See Materials and Methods for other details.

Results of Sephacryl S-200 chromatography confirm our recent results (Nakano and Ozimek, 1998) demonstrating that the majority of sialic acid in the nondialyzable fraction of sweet whey (but not acid whey containing no GMP) is from GMP aggregates comprising approximately three monomers. Glycoproteins including immunoglobulins and lactoferrin and glycolipid are probably the minor sources of sialic acid in sweet whey.

Because GMP has various biological activities including suppression of gastric secretion (Stan et al., 1983; Yvon et al., 1994) and promotion of the growth of *Bifidus* bacteria (Idota et al., 1994), determination of its concentration by monitoring sialic acid may be important in the evaluation of the properties of sweet whey and sweet whey products. Not much information is available

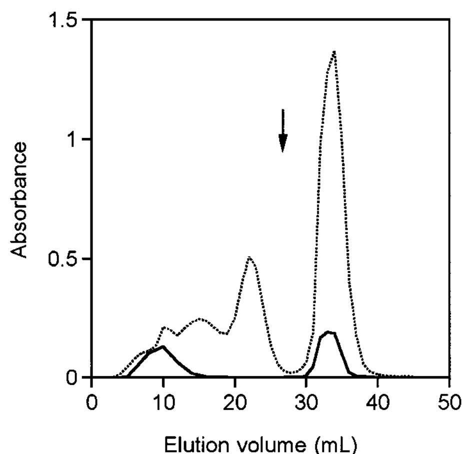

Figure 2. Representative DEAE-Sephacel chromatogram of sweet whey: (—) absorbance at 549 nm for sialic acid; (- - -) absorbance at 280 nm for protein. A portion of sweet whey was applied to a 1.5 × 4.4 cm column of DEAE-Sephacel, and fractions were collected at a flow rate of ~10 mL/h at 21 °C. The arrow shows the position of application of 1 M NaCl. The early peak fractions (elution volumes of 5−18 mL) had anomalous chromophores. See Materials and Methods for other details.

concerning variations of sialic acid contents among commercial samples of sweet whey and its fractions.

Sialic acid concentrations determined in commercial samples are shown in Table 1. The average concentration of sialic acid in sweet whey (1.8 μg/mg of dry weight), which was close to that in the sweet whey prepared from the fresh milk (see above), was 9 times lower than that in whey protein concentrate (15.9 ± 1.4 μg/mg) but 18 times higher than that in whey permeate (0.10 μg/mg). Thus, the majority of sialic acid from sweet whey is retained in whey protein concentrate as anticipated. However, in whey protein isolate, the concentration of sialic acid in sample I was similar to that in sweet whey but 10 times lower than that in sample II. The reason for this difference is unknown.

Elution profiles of sialic acid on Sephacryl S-200 were similar among all samples of sweet whey (Figure 3a,b) and whey protein concentrate (Figure 3c−g) and sample II of whey protein isolate (Figure 3i). They had major peaks (K_{av} = 0.39 ± 0.01) accounting for 76 ± 9% of recovered sialic acid. The K_{av} values were close to that (0.40) of GMP aggregate (Figure 1). Therefore, these peaks were considered to contain GMP sialic acid. This is consistent with the Sephacryl S-200 chromatography results for the sweet whey prepared from fresh milk (Figure 1). On the other hand, whey protein isolate I (Figure 3h) gave a major peak (83% of recovered sialic acid) with K_{av} = 0.45. The reason for this higher K_{av} is unknown (see below). Samples of whey permeate had major peaks (average K_{av} = 0.80) accounting for 72 and 86% of recovered sialic acid in samples I (Figure 3j) and II (Figure 3k), respectively. This may be expected to occur in these samples permeable through the ultrafiltration membrane. Nevertheless, sample I had a minor peak with K_{av} = 0.39 accounting for 22% of recovered sialic acid. Because GMP is the major source of sialic acid in sweet whey, it may be that the main sialic acid peaks found in whey protein isolate sample I (Figure 3h) and whey permeates (Figure 3j,k), eluting later than GMP aggregate, are related to the degradation products of GMP. This possibility can be checked by using antibodies that can recognize epitopes present.

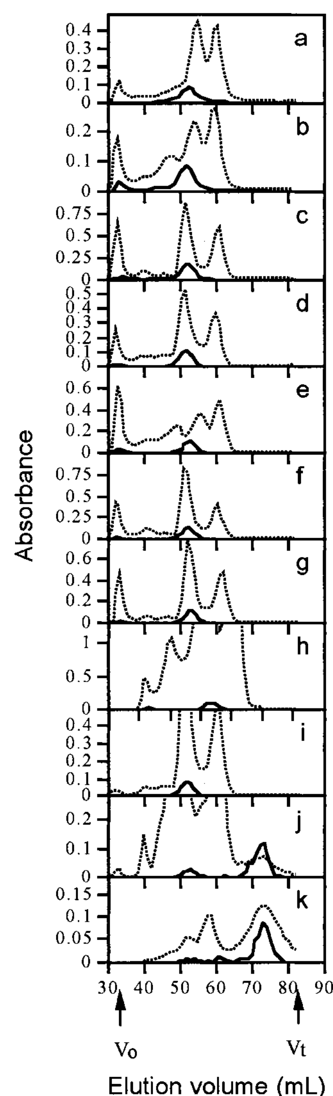

Figure 3. Chromatography of commercial samples on Sephacryl S-200: ~8−13 mg of sweet whey (samples I and II), whey protein concentrate (samples I−V), whey protein isolate sample II, and whey permeate (samples I and II) and ~117 mg of whey protein isolate sample I were separately chromatographed on the same column used for the sweet whey from fresh milk (see Figure 1): (a) sweet whey sample I; (b) sweet whey sample II; (c) whey protein concentrate sample I; (d) whey protein concentrate sample II; (e) whey protein concentrate sample III; (f) whey protein concentrate sample IV; (g) whey protein concentrate sample V; (h) whey protein isolate sample I; (i) whey protein isolate sample II; (j) whey permeate sample I; (k) whey permeate sample II. See Figure 1 for details.

The content of recovered GMP sialic acid was then calculated for samples of sweet whey and whey protein concentrate and whey protein isolate sample II. The content was highest in whey protein isolate sample II (15.8 μg/mg of dry weight) and higher in whey protein concentrate (9.5 ± 0.9 μg/mg of dry weight) than in sweet whey (1.1 μg/mg of dry weight). The content in sweet whey was ~73% of that found in the sweet whey from fresh milk (see above), suggesting that liquid sweet whey compared to commercial sweet whey powder is a more economical material for the preparation of GMP.

In conclusion, the determination of total sialic acid in sweet whey or its fraction requires dialysis, which is the most appropriate method to eliminate materials that interfere with the thiobarbituric acid reaction. Gel

chromatography on Sephacryl S-200 is a useful method to estimate the content of GMP sialic acid.

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