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Use of Epichlorohydrin-Treated Chitosan Resin as an Adsorbent to Isolate κ-Casein Glycomacropeptide from Sweet Whey

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This study was undertaken to develop a method to isolate glycomacropeptide (GMP), a bioactive compound, from sweet whey by using chitosan resins as anion exchangers. Shrimp shells were used to prepare two chitosan (polyglucosamine) resins, one with the primary amine (−NH_2) (resin A) and the other with the secondary amine (−NH−) (resin B) as the major functional group. These resins were tested as adsorbents for the isolation of GMP from sweet whey, and the results obtained were compared with those obtained with commercial anion exchangers. The most important finding in this experiment was that the GMP binding capacity of resin A was much higher than that of resin B. Resin A may be the anion exchanger to be tested for industrial scale production of GMP. Amino acid analysis of the GMP-depleted whey fraction suggests that this product can replace sweet whey as an ingredient in various food products including infant formulas, bakery products, and beverages.

KEYWORDS: Chitosan; glycomacropeptide; whey; milk; epichlorohydrin; anion exchange resin

INTRODUCTION

Bovine glycomacropeptide (GMP) found in cheese whey [or sweet whey (SW)] is a sialylated phosphorylated C-terminal peptide (residues 106−169) released from κ-casein by the action of chymosin during cheese making (*1*). GMP is known to be a biologically active compound (*2*−*4*) and is thought to be a potential ingredient for functional foods and pharmaceuticals. GMP lacking phenylalanine is also thought to be useful for the diet to maintain patients suffering from phenylketonuria, a hereditary disorder of phenylalanine metabolism (*5*). Thus, increased attention has been focused on the development of techniques to purify GMP for commercial purposes.

GMP has been purified from SW by using various techniques including ultrafiltration (*6*, *7*), gel chromatography (*8*), hydrophobic interaction chromatography (*9*), and ion exchange chromatography (*10*−*15*). Saito et al. (*16*) purified GMP using a combined method of heat treatment, ethanol precipitation, and anion exchange chromatography. Anion exchange chromatography is one of the most practical methods applicable to large scale production of GMP. By using this technique, GMP, which has a lower isoelectric point than do major whey proteins, could be separated with relatively high yield and purity (*10*, *17*).

Chitosan is a polyglucosamine polymer obtained by deacetylation of chitin, a polysaccharide found in marine invertebrates, insects, fungi, and yeasts (*18*−*21*). Industrially, chitin and chitosan are produced mainly from shrimp and crab shells, which are abundant waste products in the marine industry. Chitosan is a cationic compound insoluble in water and alkaline solutions but soluble in acidic solutions (e.g., 1.5% acetic acid). Hwang and Damodaran (*22*) used chitosan to precipitate lipids in SW as chitosan−lipid complexes formed through electrostatic interaction at pH 4.5. Bough and Landes (*23*) separated proteins from SW by precipitating chitosan−protein complexes formed at pH 6.0. There is limited information available concerning the fractionation of SW proteins and peptides using chitosan as an ion exchanger. Because chromatographic separation of GMP on an anion exchange column requires acidic conditions (*10*), chitosan must be chemically treated to obtain an adsorbent insoluble in acidic solutions. Noguchi et al. (*24*, *25*) prepared epichlorohydrin-treated chitosan resins. They tested these resins for chromatography of mandelic acid and confirmed that chitosan has an anion exchange capacity. More recently, Koide et al. (*26*) treated chitosan with ethylene diglycidyl ether to obtain a cross-linked resin, which was used for separation of phosphopeptide from trypsin digests of casein.

In this study, we have used epichlorohydrin to prepare two types of chitosan resins, one having the primary amine and the other having the secondary amine as its major functional group. These resins as anion exchangers were tested on a small scale for the isolation of GMP from a nondialyzable fraction of SW. A commercial chitosan resin and DEAE cellulose anion exchanger were also tested for the separation of GMP. The results including the yield and chemical composition of isolated GMP were compared among these anion exchangers. One of

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the chitosan resins was then tested to determine whether a high purity GMP can be isolated from commercial samples of sweet whey powder (SWP) and whey protein concentrate (WPC). These products have been used for purification of GMP by several researchers (*12*, *16*). Finally, a SW fraction left after isolating GMP, referred to as GMP-depleted whey fraction, was analyzed for amino acid composition to consider its nutritional importance.

MATERIALS AND METHODS

Materials. Shrimp shells were a gift from Dr. Buncha Ooraikul, University of Alberta. Cow milk was obtained from the Dairy Research Unit of the University of Alberta Farm. Epichlorohydrin was obtained from Sigma-Aldrich Canada Ltd. (Ontario, Canada). Chitopearl (AL-01, a chitosan resin with 0.1 mm bead diameter) was a product of Fuji Spinning Co. Ltd. (Tokyo, Japan). DEAE-Sephacel was obtained from Pharmacia-Biotech (Quebec, Canada). A sample of GMP used to determine the GMP binding capacity of chitosan resin or DEAE-Sephacel was prepared by chromatography of a nondialyzable fraction of SW on a column of DEAE-Sephacel at pH 3.0 (*17*). Two samples of either SWP containing 11% protein or WPC containing 80% protein were obtained from local suppliers.

Preparation of Chitin and Chitosan. A sample of dry shrimp shell (300 g) was pulverized using a blender and decalcified by incubating in 6 L of 2 N HCl at 4 °C for 48 h with occasional agitation. The decalcified sample was collected by centrifugation, washed with water, and deproteinized in 1.3 L of 2.5 N NaOH at room temperature (21 °C) for 72 h. Fresh NaOH solution was used in every 24 h. The insoluble residue present was washed with water and dried at 80 °C to obtain chitin (67.5 g) accounting for approximately 23% of dry shrimp shell.

Deacetylation of chitin was carried out by following a slightly modified procedure described by Noguchi et al. (*24*). To 5 g of chitin weighed in a glass conical flask, 100 mL of 60% NaOH was added. The mixture was stirred and heated at 150−160 °C for 1 h on a hot plate with a magnetic stirrer. After alkali treatment, the product collected was washed with water and dried at 80 °C to obtain chitosan (average 3.7 g) accounting for 74% dry weight of chitin. A portion of the final product of chitosan was tested to confirm that it is soluble in 1.5% acetic acid.

Preparation of Chitosan Resins. The chitosan resin with the primary amine was prepared by following the procedure described by Noguchi et al. (*25*). To 5 g of chitin, 50 mL of 10 N NaOH was added to prepare a suspension of alkaline chitin. After incubation at 4 °C overnight, the mixture was centrifuged at 20000*g* and 4 °C. The residue obtained was then mixed with 5 g (= 4.23 mL) of epichlorohydrin in 50 mL of water and incubated at 4 °C for 48 h with occasional agitation. The product obtained after centrifugation followed by washing with water was the epichlorohydrin-treated chitin resin with its dry weight averaging 4.3 g, which corresponded to 86% of dry chitin. In this product, epichlorohydrin is attached through the OH group of chitin with the N-acetylglucosamine kept intact. The portion of the epichlorohydrin-treated chitin (4 g) was then deacetylated by heating in 60% NaOH at 150−160 °C for 1 h to obtain the chitosan resin with the primary amine, which was referred to as resin A (average 2.9 g accounting for 73% of the initial weight of epichlorohydrin-treated chitin resin).

The chitosan resin with the primary and secondary amines was prepared as described by Noguchi et al. (*24*). To a suspension prepared with 4 g of shrimp chitosan and 40 mL of water, 2.32 g of epichlorohydrin was added, and the mixture was stirred and heated at 95−100 °C for 1 h on a hot plate. Water was added to the mixture to maintain its volume when water was lost due to evaporation. After it was heated, the mixture was cooled to room temperature. To this preparation, 28 mL of 1 N NaOH was added and the mixture was heated at 120 °C for 2 h to obtain the chitosan resin, in which epichlorohydrin is attached through the NH_2 as well as OH group to form the secondary amine as the major functional group. The resin was then washed in water and dried at 80 °C overnight to obtain an average of 4.1 g of the final product, referred to as resin B, accounting for 103% of the initial weight of chitosan.

Both resins A and B were tested to confirm that they are insoluble in 1.5% acetic acid. Each resin was finely powdered by using a mortar and pestle, and powders that passed through a 200 mesh screen (75 μm opening) were used as an anion exchange resin for the adsorption of GMP.

Preparation of SW. A sample of fresh bovine milk was hydrolyzed with chymosin as previously described (*4*). The hydrolysate obtained was then centrifuged at 20000*g* and 4 °C to remove fat and protein precipitate. The SW obtained as a supernatant was transferred into dialysis tubes with a 6000−8000 molecular weight cutoff and dialyzed in running tap water for 24 h and then for another 24 h in deionized water at 4 °C. For samples of SWP, a portion of each sample was mixed with 20 volumes of water, and the mixture was dialyzed as described above. The product retained in the dialysis tube (nondialyzable fraction) was freeze-dried and then stored at 4 °C until used for the isolation of GMP. The removal of salts by dialysis is an important process to maximize the yield of GMP adsorbed on the anion exchanger (*10*). Samples of WPC with relatively low contents of dialyzable material (<7% of dry weight, T. Nakano and L. Ozimek, unpublished data) were not dialyzed. The dry weight of nondialyzable fraction accounted for 0.54% (w/v) for the SW prepared from the fresh milk and 11.3 and 13.0% (w/w) for SWP samples I and II, respectively.

Isolation of GMP. A small scale batch method separation of GMP was carried out at 21 °C and different pH values (2.2, 2.5, 2.8, 3.0, 3.5, and 4.0) by using the following procedure. Approximately 50 mg of nondialyzable fraction of SW or WPC was mixed with 10 mL of water. The mixture was adjusted to an appropriate pH with HCl and centrifuged at 20000*g* for 25 min. After centrifugation, the supernatant collected was mixed in a beaker with anion exchanger (~30 mg dry weight) previously washed with water having an appropriate pH. The mixture was left for 1 h with occasional agitation. The pH of the mixture was checked and maintained constant during the incubation period. The mixture was then transferred to a 15 mL conical bottom plastic centrifuge tube and centrifuged. The supernatant obtained was collected in the first of a series of tubes. The anion exchanger collected in the bottom of centrifuge tube was transferred to a 1.5 mL microcentrifuge tube, washed by vortexing with 1 mL of water, and centrifuged at 12000*g* for 1 min. The supernatant was collected in the second tube. After a series of 15 washes, GMP adsorbed on an anion exchanger was extracted (by a series of extractions) with 20 mL of 2 M NaCl. The anion exchanger was then washed with water and ready for use again. The pH of the water and 2 M NaCl used was previously adjusted to an appropriate value. The use of acidic water and NaCl without buffer containing inappropriate chemicals for food preparation was thought to be safe for separation of GMP for human consumption. In this case, a batch procedure (instead of column chromatography) was practical to hold the pH value constant in the mixture of sample and resin by checking and adjusting it. Use of each anion exchanger several times showed no appreciable change in its GMP binding capacity. All supernatants collected were monitored for absorbance at 230 nm, and those containing desorbed or adsorbed materials were separately pooled, dialyzed in water, and freeze-dried to obtain two fractions. One contained GMP adsorbed on anion exchanger and eluted from it (GMP fraction) and the other contained non-GMP components desorbed from the anion exchanger (GMP-depleted whey fraction).

Comparison of GMP Binding Capacity. A mixture of GMP (~35 mg, see Materials) and anion exchanger (~10 mg) was incubated at pH 3.0 and 21 °C for 1 h with occasional agitation. After incubation, the mixture was centrifuged, and the anion exchanger collected in the bottom of centrifuge tube was washed with water having a pH of 3.0. The GMP adsorbed on the anion exchanger was then eluted with 2 M NaCl. All supernatants collected by a series of vortexing and centrifugation were monitored for GMP by measuring absorbance at 230 nm. The content of GMP that was adsorbed on anion exchanger and eluted with 2 M NaCl was calculated using the following formula: $(A/A_{GMP}) \times V$, where A is the absorbance of supernatant containing eluted GMP and A_{GMP} is the absorbance of the solution containing 1 mg GMP/mL and V is the volume (mL) of supernatant containing

Table 1. Analysis of Acetic Acid Recovered from Chitin, Chitosan, and Chitosan Resin Samples after Acid Hydrolysis[a]

sample	μg/mg dry weight
chitin[b]	270.0
chitosan	52.0
resin A	41.5
resin B	50.2
chitopearl	55.5

[a] Values presented are the averages from two experiments, in that the results obtained in the first experiment were similar to those in the second experiment. [b] Used to prepare resins A and B.

eluted GMP. The amount of GMP adsorbed on anion exchanger was calculated and compared among chitosan resins and DEAE-Sephacel.

Analytical Methods. The content of the acetyl group in a sample of chitin, chitosan, or chitosan resin was estimated by determining the content of acetic acid recovered after acid hydrolysis from N-acetylglucosamine remaining in sample. Approximately 20 mg of sample was hydrolyzed in 0.5 mL of 6 N HCl at 110 °C overnight. The portion of the hydrolysate (1−3 μL) was used for the determination of acetic acid by gas chromatography. A Varian 3400 gas chromatograph equipped with a Varian 8100 auto sampler and FID detector was used with a Stabilwax-DA column (30 mm × 0.53 mm inside diameter, Resteck Corp., Bellefonte, PA). The initial column temperature was 120 °C. The temperature was increased by 10 °C/min to the final temperature of 170 °C, which was held for 5 min.

The sialic acid content in the GMP fraction was determined by the thiobarbituric acid reaction (*27*) after hydrolysis of samples in 0.1 N sulfuric acid at 80 °C for 1 h. The chromophore formed was extracted using 1-propanol (*28*) instead of cyclohexanone (*27*). For amino acid analysis, samples (approximately 1 mg) were hydrolyzed under nitrogen at 110 °C for 24 h in glass distilled 6 N HCl containing 0.1% (w/v) phenol. The portion of the hydrolysate was then determined for amino acid composition using a Beckman model 6300 amino acid analyzer. Cysteine and tryptophan were not determined.

RESULTS AND DISCUSSION

Table 1 shows the contents of acetic acid recovered from chitin, chitosan, and chitosan resin samples after acid hydrolysis. The acetic acid contents were similar among the samples of chitosan and chitosan resin averaging 49.8 μg/mg of dry weight, which was approximately 18% of the amount of acetic acid recovered from chitin (with a high concentration of N-acetylglucosamine). This confirms that the degree of deacetylation is sufficiently high in the three resins including resins A and B and chitopearl and that resin A prepared by deacetylation of epichlorohydrin-treated chitin resin contains the primary amine as intended (see Materials and Methods). No analysis was undertaken to estimate the content of primary or secondary amines in this study. However, Noguchi et al. (*25*) analyzed nitrogen in their preparation of epichlorohydrin-treated chitosan resin with the primary amine and reported that the resin contains the primary amino nitrogen only with no secondary or tertiary amino nitrogen present. Noguchi et al. (*24*) also analyzed nitrogen in their epichlorohydrin-treated chitosan resin with the primary and secondary amines and reported that most (81.9%) of the nitrogen in their resin was in the secondary amino nitrogen.

Figure 1 shows the yields of the GMP fraction obtained from a nondialyzable fraction of bovine SW by using resin A at different pH values. The phenylalanine content (reflecting the amount of impurity in the GMP fraction) is also shown. Both parameters were the lowest at pH 2.2 and increased with increasing pH value. Similar observations were obtained with

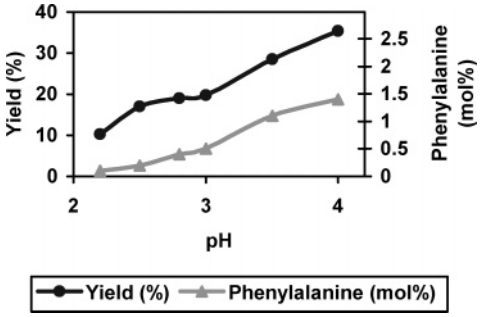

Figure 1. Effect of pH on the yield of bovine GMP fraction and its phenylalanine content. Data shown in the diagram were obtained by using resin A. The yield of GMP was calculated as a percent of dry weight of the sample used for separation of GMP. Values plotted are the averages from two experiments, in that the results obtained in the first experiment were similar to those in the second experiment.

the other anion exchangers including resin B, chitopearl, and DEAE-Sephacel (results not shown). Therefore, the purity of GMP was inversely related to the yield of this compound. The optimum pH for the separation of GMP on these anion exchangers is approximately 3. This value is within the range (2.5−4.0) reported previously for purification of GMP by DEAE-cellulose chromatography (*10*).

The yield and chemical composition of GMP fraction obtained with the four anion exchangers at pH 3.0 are given in **Table 2**. The yield was similar among the anion exchangers tested and averaged 20.6% of dry weight of nondialyzable fraction of SW. This value, corresponding to 0.11% (w/v) of SW (see above for the yield of nondialyzable fraction of SW), is comparable to the range of GMP content (1.1−1.5 g/L of SW) reported in the literature (*2*, *29*). Amino acid compositions and sialic acid concentrations (averaging 52.3 μg/mg dry weight) were also similar among the anion exchangers. Amino acid compositions showed the presence of traces (each <1 mol %) of tyrosine, phenylalanine, and histidine (amino acids absent in GMP). Tyrosine was undetectable in GMP fractions obtained by using resin A and chitopearl. Amino acid compositions are in general comparable to those in GMP purified previously by anion exchange chromatography (see **Table 2**). Sialic acid concentrations are within the range (5−189 μg) reported by Kawakami et al. (*30*). These findings confirm that GMP can be separated from SW by using the epichlorohydrin-treated chitosan resins as well as DEAE-cellulose. The purity of GMP isolated using chitosan resins may be sufficient for partial replacement of amino acids in commercial diets for phenylketonuria patients.

Table 3 shows the GMP binding capacity of anion exchanger, which is an important factor affecting the isolation cost of GMP. The binding capacity expressed as mg GMP per mg dry weight of anion exchanger was approximately 2.5-fold higher in resin A than in resin B and relatively constant between resin A and chitopearl. The binding capacity of DEAE-Sephacel was 1.8-fold lower than that of resin A but 1.4-fold higher than that of resin B. Resin A, with a higher capacity than resin B, was then tested for separation of GMP from commercial samples of SWP and WPC.

Table 4 shows the yield and chemical composition of GMP fraction from a nondialyzable fraction of SWP and WPC. The yield of the GMP fraction was similar between samples I and II from either product. In the nondialyzable fraction of SWP, the yield averaged 15.1% of dry weight, which corresponded to approximately 2.0 and 3.2% of dry weight of SWP samples I and II, respectively. These values are fairly comparable to the yield 1.8−2.5% calculated from the value 1.1−1.5 g GMP per

Table 2. Analysis of GMP Fractions Obtained Using Different Anion Exchangers[a]

	resin A	resin B	chitopearl	DEAE-Sephacel	GMP I[b]	GMP II[c]	GMP VA[d]	GMP VB[e]
yield[f]	19.8	21.7	20.1	20.8				
amino acid				mol %				
asx	7.9	7.9	8.1	7.9	8.3	8.6	7.8	6.3
thr	13.7	14.7	12.9	14.0	14.0	13.3	18.8	17.2
ser	14.4	12.1	14.6	12.9	8.5	9.2	9.4	9.4
glx	20.3	19.1	21.0	19.0	17.7	20.0	15.6	15.6
gly	2.3	2.1	2.5	2.2	2.5	1.9	1.6	1.6
ala	6.1	5.9	5.6	5.6	7.5	6.5	7.8	9.4
val	7.0	7.2	7.0	7.1	7.4	8.3	9.4	9.4
met	0.5	0.9	0.5	1.1	0.5	†[g]	1.6	1.6
ile	8.8	8.6	8.9	8.5	9.7	9.0	9.4	10.9
leu	3.5	3.9	4.1	4.2	3.7	4.0	1.6	1.6
tyr	ND[h]	0.3	ND	0.3	ND	ND	0	0
phe	0.5	0.6	0.3	0.5	0.7	0.8	0	0
his	0.4	0.5	0.4	0.5	1.3	0.6	0	0
lys	3.6	4.7	3.3	4.5	5.9	5.4	4.7	4.7
arg	1.1	1.3	1.3	1.6	0.9	1.0	0	0
pro	9.8	10.1	9.5	10.3	11.4	11.1	12.5	12.5
SA[i]	55.1	52.1	50.6	51.3				

[a] Values presented are the averages from two experiments, in that the results obtained in the first experiment were similar to those in the second experiment. [b,c] Purified by anion exchange chromatography by Tanimoto et al. (*33*) and Nakano and Ozimek (*17*), respectively. Amino acid contents were calculated from the data reported by these authors. [d,e] Bovine GMP variants A and B, respectively. Amino acid contents are based on the primary structure of variant A or B (*1*). [f] Percent of dry weight of sample used for separation of GMP. [g] Not determined. [h] Not detected. [i] Sialic acid (μg/mg dry weight).

Table 3. Comparison of GMP Binding Capacity among Anion Exchangers[a]

anion exchanger	mg GMP/mg dry weight of anion exchanger
resin A	2.18
resin B	0.87
chitopearl	2.29
DEAE-Sephacel	1.19

[a] The mixture of GMP and anion exchanger was incubated at pH 3.0. Values presented are the averages from two experiments, in that the results obtained in the first experiment were similar to those in the second experiment. See the Materials and Methods for other details.

L of SW (*2*, *29*) assuming that SW contains 6.1% dry matter (*31*). The yield of GMP fraction from WPC, averaging 11.9% (w/w), was apparently lower than that found in the nondialysable fraction of SWP (see above). A small amount of lactose present in the samples (average 4% of dry weight according to the suppliers' information) may be one of the factors causing the underestimation of the yield. It is unknown whether the lower yield is due to the loss of GMP or its extractability during preparation of WPC. All GMP fractions from either product contained traces (<1 mol %) of tyrosine, phenylalanine, and histidine (amino acids not found in GMP). This suggests that resin A can be used for the isolation of GMP from SWP or WPC with a trace of phenylalanine. Amino acid compositions were similar, in general, among the samples from SWP and WPC sample I and were comparable to those of nondialyzable fractions of SW from fresh milk (**Table 2**). The amino acid composition in WPC sample II was, however, different from that found in the remaining samples, which had higher contents of asparagine/aspartic acid, threonine, and serine and lower contents of alanine, valine, isoleucine, and proline than did WPC sample II. The sialic acid concentration in the GMP fraction was approximately 2-fold lower in SWP sample I than in the remaining samples having an average concentration of 65.2 μg/mg. The reason for these differences observed in the amino acid composition and sialic acid concentration is unknown. Because most sialic acid in SW is found in GMP, sialic acid assay has been suggested to be useful to detect SW present as an ingredient in processed foods. However, the variation found in the sialic acid concentration herein suggests that detection of SW in processed foods by analyzing sialic acid is possible qualitatively but not quantitatively.

Table 4. Analysis of GMP Fractions from SWP and WPC[a]

	SWP I[b]	SWP II[c]	WPC I[d]	WPC II[e]
yield[f]	15.0	15.1	12.6	11.2
amino acid		mol %		
asx	7.7	7.6	7.2	5.0
thr	15.4	14.9	16.1	12.1
ser	11.5	11.5	11.3	9.1
glx	16.8	17.3	16.6	16.9
gly	2.3	2.1	2.0	2.1
ala	7.3	6.8	7.8	8.6
val	7.8	7.7	8.1	9.5
met	1.7	1.3	1.3	1.4
ile	8.4	8.3	8.9	10.2
leu	3.3	3.4	3.0	3.6
tyr	0.4	0.4	0.3	0.3
phe	0.8	0.9	0.7	0.8
his	0.6	0.6	0.4	0.5
lys	3.7	4.7	4.5	5.0
arg	1.3	1.2	0.7	1.0
pro	11.0	11.1	11.0	13.8
SA[g]	35.0	62.0	69.0	64.5

[a] Resin A was mixed with SWP or WPC, and the mixture was incubated at pH 3.0. Values presented are the averages from two experiments, in that the results obtained in the first experiment were similar to those in the second experiment. See the Materials and Methods for other details. [b,c] Commercial SWP samples obtained from two suppliers. [d,e] Commercial WPC samples obtained from two suppliers. [f] Percent of dry weight of sample used for separation of GMP. [g] Sialic acid (μg/mg dry weight).

After GMP was separated, we obtained a GMP-depleted whey fraction containing proteins desorbed from the anion exchangers. The yield of the fraction was estimated from the difference between the amount of GMP collected and that of the SW sample used to isolate GMP. The average yield was 79.4% (w/w) of the nondialyzable fraction of SW from fresh milk, which corresponded to 0.43% (w/v) of liquid SW. The yield of GMP-depleted whey fraction derived from the commercial

Table 5. Amino Acid Compositions of GMP-Depleted Whey Fractions from SW, SWP, and WPC, and Those of SW and Acid Whey

	mol %				
amino acid	SW[a]	SWP[b]	WPC[c]	SW[d]	acid whey[e]
asx	10.6 ± 0.2	10.6	10.4	9.9	9.3
thr	6.9 ± 0.4	7.4	7.7	8.2	6.2
ser	6.3 ± 0.2	7.4	7.6	6.4	8.1
glx	15.4 ± 0.3	14.9	14.6	16.1	15.4
gly	3.0 ± 0.1	3.2	3.4	2.8	3.4
ala	7.0 ± 0.1	7.1	7.3	6.9	6.6
val	6.1 ± 0.0	6.1	6.2	7.2	6.6
met	2.2 ± 0.4	1.8	1.7	1.3	1.7
ile	5.5 ± 0.1	5.8	5.8	7.3	5.2
leu	11.7 ± 0.4	11.7	11.3	9.9	11.2
tyr	2.5 ± 0.2	2.9	2.7	1.9	2.9
phe	2.9 ± 0.1	3.2	3.1	2.5	3.3
his	2.2 ± 0.4	1.9	1.7	1.5	2.1
lys	9.0 ± 0.2	7.4	7.9	8.4	7.8
arg	1.6 ± 0.0	1.9	2.0	1.7	2.7
pro	7.1 ± 0.2	6.7	6.6	8.0	7.5

[a] The value presented is the mean ± standard deviation obtained from the four determinations each in GMP-depleted whey fraction from resin A, resin B, chitopearl, or DEAE-Sephacel. [b,c] The value presented is the mean of the two determinations each in GMP-depleted whey fraction from SWP or WPC. [d] A single sample of nondialyzable fraction of SW used in this study to isolate GMP (see Materials and Materials). [e] A single sample of acid whey prepared as described previously (*34*).

samples of SWP and WPC averaged 84.9 and 88.1% (w/w), respectively. Amino acid analysis (**Table 5**) showed that the GMP-depleted whey fraction has high contents of glutamine/glutamic acid, leucine, and asparagine/aspartic acid and that the threonine content in the GMP-depleted whey fraction is lower than that in SW but higher than that in acid whey. It has been reported that the GMP with a high content of threonine is the major factor causing hyperthreoninemia, which is a frequent abnormality found in preterm infants fed SW-based formulas, and that feeding acid whey-based formula free of GMP prevents hyperthreoninemia (*32*). However, the supply of acid whey, a byproduct of casein and cottage cheese production, is much less than the supply of SW. It is interesting to determine whether the GMP-depleted whey fraction is as effective as acid whey to prevent hyperthreoninemia. The GMP-depleted whey fraction could also be used to replace SW as an ingredient in various food products such as bakery products and beverages. It appears that separation of SW into GMP and GMP-depleted whey fractions by using anion exchangers is a significant useful process for the dairy industry.

To our knowledge, this is the first report of application of chitosan epichlorohydrin resin to the separation of GMP. We now suggest that resin A with the primary amine as the functional group has a higher GMP binding capacity than does resin B with the secondary amine as the major functional group and that separation of GMP with resin A is the technique to be scaled up. Resin A can be prepared with a relatively simple low cost method, in that chitin isolated from shrimp shell is treated with epichlorohydrin to form a chitin epichlorohydrin resin, where the N-acetylglucosamine is kept intact. This resin is subsequently deacetylated by alkaline hydrolysis to obtain the anion exchange resin with a high concentration of NH_2 group. Resin A can be used for the adsorption of various proteins, peptides other than GMP, and carbohydrates as well as GMP.

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