Selective Removal of Phenylalanine Impurities from Commercial $\kappa$-Casein Glycomacropeptide by Anion Exchange Chromatography

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Abstract

Bovine $\kappa$-casein glycomacropeptide (GMP) found in sweet whey is a 64 amino acid residue phosphorylated glycopeptide. Because it lacks aromatic amino acids including phenylalanine, GMP is thought to be an important dietary source of amino acids for patients suffering from phenylketonuria. There is, however, very little information available concerning preparation of phenylalanine-free GMP for human consumption. This study was, therefore, undertaken to remove phenylalanine containing impurities from commercially available crude GMP by anion exchange chromatography on diethylaminoethyl (DEAE)-Sephacel. The results demonstrated that phenylalanine containing proteins or peptides do not bind to the column, while most GMP accounting for 93% of total recovered sialic acid can bind to the column. The purified GMP, which accounted for average 43% of dry weight of crude GMP, contained undetectable level of phenylalanine. Carbohydrate analyses and cellulose acetate electrophoresis showed that the purified GMP is a product with high sialic acid content (average 15.5% dry weight). Gel filtration chromatography on Sephacryl S-100 and size exclusion HPLC on Superdex 75 confirmed our previous findings that GMP monomers form aggregates and elute as a single peak with its elution volume close to the elution volume of dimeric $\beta$-lactoglobulin (36.6 kDa). It was concluded that the crude preparation of GMP can be highly refined by selectively removing phenylalanine impurities using DEAE-Sephacel chromatography.

Keywords: $\kappa$-Casein glycomacropeptide; Caseinomacropeptide; Sialic acid; Anion exchange chromatography; DEAE-Sephalce

Introduction

Glycomacropeptide (GMP) found in sweet whey (or cheese whey) from cow’s milk is a 64 amino acid residue C-terminal phosphorylated glycopeptide (residues 106-169) released from $\kappa$-casein by the action of chymosin, which catalyzes cleavage between residues 105 (phenylalanine) and 106 (methionine) of $\kappa$-casein during cheese making [1-5]. GMP contains varying amounts of carbohydrates including N-acetylgalactosamine, galactose and N-acetylneuraminic acid (sialic acid) [6]. GMP is known to have various biological activities (e.g. protection against toxins, bacteria, and viruses, and modulation of immune responses [3]), and is thought to be a potential ingredient for functional foods and pharmaceuticals. GMP, which lacks aromatic amino acids (phenylalanine, tyrosine, and tryptophan), is thought to be suitable for the source of dietary amino acids for patients suffering from phenylketonuria (PKU), a hereditary disorder of phenylalanine metabolism causing mental retardation [7,8]. Thus, much attention has been given to the development of techniques to prepare high purity GMP.

Olieman and van Riel [9] by using trichloroacetic acid treatment and reversed-phase HPLC isolated GMP from sweet whey, and reported absence of phenylalanine in their final preparation. Nakano et al. [10] also reported that GMP fraction from sweet whey prepared by trichloroacetic acid treatment followed by gel filtration chromatography contained undetectable level of phenylalanine. However, the use of trichloroacetic acid is not suitable for production of GMP for human consumption. For preparation of GMP as a food, ion exchange is one of the common techniques, in that GMP having an isoelectric point (pI) < 3.8 [11], which is lower than the pI (> 4.1) of major whey proteins ($\beta$-lactoglobulin, $\alpha$-lactalbumin, serum albumin, immunoglobulins etc.) [1], can be separated from whey proteins by the difference of pI. GMP fractions obtained by ion exchange techniques contain traces of aromatic amino acids as contaminants. For example, Nakano and Ozimek [12] reported that GMP isolated from sweet whey using anion exchange chromatography on diethylaminoethyl (DEAE)-Sephacel contained less than 1 residue of each of phenylalanine, histidine and arginine per peptide. Similarly, it has been reported that GMP isolated from sweet whey or whey protein concentrate by anion exchange techniques contained low but detectable levels of aromatic amino acids including phenylalanine, and other amino acids not found in GMP [11,13,14]. More recently, LaClair et al. [7], in their experiment of PKU diet preparation, reported that the phenylalanine concentration (5 mg/g of product) in a commercially available GMP (Davisco Foods International, Inc. USA.) is too high, and thus, they refined the product by using cation exchange chromatography to reduce phenylalanine level to 2.7 mg/g protein equivalent (43% reduction). This suggests that purification of GMP by ion exchange chromatography without having contaminating amino acids including phenylalanine is very difficult, although the following information is available in the literature. Léonil and Mollé [15] isolated GMP from sweet whey using cation exchange HPLC on a Mono-S column, whereas Saito et al. [16] purified GMP by using ethanol precipitation followed by DEAE-Toyopearl anion exchange chromatography. Both groups of researchers reported preparation of GMP with no contaminating amino acids including histidine, tyrosine, arginine, and phenylalanine. However, these results must be interpreted carefully. Amino acid analysis in either study showed recovery of less than 64 residues/peptide [i.e. 59 and 60 residues/peptide, each calculated from the data of Léonil and Mollé...
the molybdate-vanadate reagent [24] with potassium dihydrogen concentrations in crude and purified GMP were determined by using indole reaction [23] after hydrolysis of samples in 4 M HCl at 100ºC acid. Galactose was determined by the anthrone reaction [22] using absorbance with the absorbance of a known concentration of sialic or purified preparation of GMP was calculated by comparison of its thiobarbituric acid reaction is a hazardous chemical too difficult to undertake to determine whether commercially available GMP can be refined by anion exchange chromatography with high reproducibility to provide GMP with no contaminating amino acids.

Materials and Methods

Materials

A commercial sample of crude GMP prepared using ion exchange technique was obtained from Davisco Foods International, Inc., Eden Prairie, MN., USA. DEAE-Sephacel and Superdex G-75 were products of GE Healthcare, Baie d’Urée, PQ, Canada. Sialic acid (N-acetylmuraminic acid from sheep submaxillarily glands), galactose, galactosamine-HCl, and Sephacryl S-100-HR were obtained from Sigma-Aldrich, Canada Ltd., Mississauga, ON, Canada. Cellulose acetate strips (Sepaphore III, 2.5 cm × 15.2 cm) were obtained from Pall Corporation, Ann Arbor MI, USA. Malachite green was obtained from Difco Laboratories, Detroit, MI., USA.

Anion exchange chromatography

To a sample (~0.2 g) of crude GMP, 40 ml of water was added. This amount of water was used for the convenience of pH measurement and centrifugation. The mixture was adjusted to pH 3.0 with 1 M HCl. A small amount of precipitate formed was removed by centrifugation at 20,000 × g and 21ºC for 20 min, and a supernatant obtained was applied to a 1.5 cm × 6.2 cm column of DEAE-Sephacel equilibrated with water, adjusted to pH 3.0 with 1 M HCl. Immediately after application of sample, the column was washed with approximately 40 ml of water adjusted to pH 3.0, and then eluted with a linear gradient formed from 45 ml of water and 45 ml of 1 M NaCl, both adjusted to pH 3.0. Fractions (~2 ml) collected at a flow rate of 23 ml/h were monitored for ultra violet (UV) absorbance at 210 and 230 nm for peptide amide bond, and 280 nm for protein, which is dependent mainly on the amounts of tyrosine and tryptophan [17]. Fractions were also monitored for carbohydrates (sialic acid, galactose, and galactosamine), and phosphorus. The recovery of GMP was estimated by monitoring sialic acid, a marker compound specific to sialylated GMP [18]. The major sialic acid containing fractions and those containing components unadsorbed to DEAE-Sephacel were separately pooled, exhaustively dialyzed in water, and freeze-dried for further studies. This experiment was repeated six times.

Analytical methods

The sialic acid content was determined by the modified thiobarbituric acid reaction [19,20], in that 1-propanol instead of cyclohexanone [21] was used to extract chromophore formed during reaction. Cyclohexanone used by Warren [21] in the original thiobarbituric acid reaction is a hazardous chemical too difficult to handle in assaying many samples. The absorbance of chromatophore was read at 549 nm, and the concentration of sialic acid in the crude or purified preparation of GMP was calculated by comparison of its absorbance with the absorbance of a known concentration of sialic acid. Galactose was determined by the anthrone reaction [22] using galactose as a standard, and galactosamine was determined by the indole reaction [23] after hydrolysis of samples in 4 M HCl at 100ºC for 4 h. The galactosamine value obtained was corrected for hydrolytic loss of 10% determined on standard galactosamine-HCl. Phosphorus concentrations in crude and purified GMP were determined by using the molybdate-vanadate reagent [24] with potassium dihydrogen phosphate as a standard phosphate. Fractions obtained after anion exchange chromatography or cellulose acetate electrophoresis was also monitored for phosphorus by using the malachite green dye binding method [25].

For analysis of amino acids except tryptophan, a sample (~3 mg) was hydrolyzed in 3 ml of 6 M HCl in the presence of nitrogen at 110ºC for 24 h. For tryptophan analysis, a sample (~3 mg) was first dissolved in 3 ml of 4.2 M NaOH, to which 0.75 ml of 2 M pyrogallol was added, and the mixture was hydrolyzed in the presence of nitrogen at 110ºC for 20 h. Amino acids in both the acid and alkali hydrolysates were derivatized using the o-phthalaldehyde method [26,27] using a fluorophore reagent prepared by dissolving 0.25 g of o-phthalaldehyde in 6 ml of methanol followed by the addition of 56 ml of 0.04 M sodium borate buffer, pH 9.5, 0.25 ml of 2-mercaptoethanol, and 2 ml of Brij 35. Chromatographic analysis of the derivatized amino acids was carried out using a Supelcosil 3 micron LC-18 reverse phase column (4.6 mm × 150 mm, Supelco) with a Varian Fluorichrom fluorescence detector (excitation 340 nm and emission 450 nm).

Size exclusion HPLC

A 20 µL of sample containing 100 µg of purified GMP, crude GMP or the product unadsorbed on DEAE-Sephacel was applied to 1 cm × 30 cm column of Superdex 75 10/300 GL equilibrated and eluted with 0.05 M sodium phosphate-0.15 M NaCl, pH 7.0. The eluate was monitored for peptide by measuring absorbance at 214 nm.

Cellulose acetate electrophoresis

Electrophoresis of GMP on cellulose acetate strips was carried out in 0.1 M pyridine-1.2 M acetic acid, pH 3.5 [20]. After electrophoresis, GMP was located by monitoring sialic acid and phosphorus in serial fractions obtained from the cellulose acetate strip [20].

Gel filtration chromatography

Molecular size of GMP purified using DEAE-Sephacel anion exchange chromatography was examined using gel filtration chromatography on Sephacryl S-100-HR. Two columns with similar size were prepared. The first one (0.9 cm × 57 cm) was equilibrated and eluted with 0.05 M phosphate-0.15 M NaCl, pH 7.0, and the second one (0.9 cm × 58.5 cm) was equilibrated and eluted with 6 M guanidine hydrochloride-0.1 M sodium acetate, pH 7.0. In each case, approximately 2.5 mg portion of GMP sample was chromatographed at a flow rate of 9 ml/h, and elution patterns of GMP were compared between the two columns.

Results and Discussion

The crude GMP suspension showed an average pH value of 6.4 with relatively low turbidity [0.052 ± 0.001 (standard deviation, SD, n =6)] against water at wavelength 500 nm]. Its turbidity increased 2.3 times (0.120 ± 0.006) when the pH was adjusted to 3.0. After centrifugation, the supernatant collected was applied to the anion exchange column of DEAE-Sephacel, whereas the precipitate obtained, which accounted for 1.6 ± 0.4% dry weight of crude GMP, was discarded.

A representative DEAE-Sephacel chromatogram for the crude GMP is shown in Figure 1. A small proportion of sialic acid (corresponding to 6.8 ± 5.7% of total recovered sialic acid) failed to bind to the anion exchanger, whereas most (93.2 ± 5.7%) of the recovered sialic acid was adsorbed on the column, and eluted at 0.3-0.6 M NaCl (Figure 1A). The sialic acid found in the unadsorbed component (fractions 9-33, Figure 1A) was confirmed to be GMP sialic acid (but unlikely sialic acid
from whey proteins including α-lactalbumin and immunoglobulin) by cellulose acetate electrophoresis (data not shown). The major sialic acid peak fractions (57-68) (Figure 1A) also contained peptide, galactose, galactosamine, and phosphorus, each showing its elution position identical to that of sialic acid (Figure 1B), reflecting the structure of GMP as phosphorylated peptide to which sialylated oligosaccharides are covalently attached [1-5]. The product adsorbed on the column (referred to as purified GMP), and the product unadsorbed on the column (unadsorbed product) accounted for 42.6 ± 5.9% and 36.0 ± 10.0%, respectively, of dry weight of crude GMP sample applied to the column. The apparently larger peak area of UV absorbance (at 210 nm and 230 nm) seen in the unadsorbed than in the adsorbed product (Figure 1A), which apparently does not reflect the difference in the recovered dry weight between the two products as reported above, is likely due to the higher concentration of non-peptide component (i.e. carbohydrate) in the latter (see below).

Amino acid analysis (Table 1) showed that the small amount of phenylalanine, present in the crude GMP, was also found in the unadsorbed product, but not in the purified GMP. This indicated that phenylalanine containing protein or peptide did not bind to the anion exchanger. The amino acid composition of the purified GMP was similar among the six experiments, suggesting that the separation of GMP with anion exchange chromatography. The amino acid composition of the purified GMP was similar among the six experiments, suggesting that the separation of GMP with anion exchange chromatography.

|                | Crude GMP | Product adsorbed on the column (purified GMP) | Product unadsorbed on the column |
|----------------|-----------|---------------------------------------------|---------------------------------|
| Asx            | 0.50 ± 0.04 | 0.47 ± 0.05                                | 0.62 ± 0.04                     |
| Glx            | 1.10       | 1.05 ± 0.08                                | 1.34 ± 0.10                     |
| Ser            | 0.66       | 0.57 ± 0.08                                | 0.72 ± 0.10                     |
| Gly            | 0.12       | 0.11 ± 0.01                                | 0.13 ± 0.01                     |
| Thr            | 1.17       | 1.17 ± 0.10                                | 1.28 ± 0.07                     |
| Arg            | 0.02       | 0.01 ± 0.00                                | 0.03 ± 0.01                     |
| Ala            | 0.53       | 0.52 ± 0.04                                | 0.74 ± 0.25                     |
| Val            | 0.50       | 0.54 ± 0.02                                | 0.64 ± 0.05                     |
| Ile            | 0.58       | 0.60 ± 0.04                                | 0.69 ± 0.08                     |
| Leu            | 0.15       | 0.12 ± 0.01                                | 0.24 ± 0.03                     |
| Lys            | 0.27       | 0.29 ± 0.02                                | 0.40 ± 0.02                     |
| Tyr            | 0.06       | nd                                          | 0.02 ± 0.02                     |
| Phe            | 0.03       | nd                                          | 0.04 ± 0.01                     |
| Trp            | < 0.01     | < 0.01                                      | < 0.01                          |
| Absorbance at 280 nm for 1 mg/mL solution  | 0.061 ± 0.00 | 0.014 ± 0.001                              | 0.072 ± 0.030                   |

Proline, methionine, and cysteine were not determined.

*Average of two determinations.

*Mean ± SD (n = 6).

nd: Not detected.

*Each sample was filtered through 0.22 µm membrane before measurement of absorbance, and absorbance values are shown as mean ± SD (n = 6).

Table 1: Amino acid concentrations (µmole/mg) and UV absorbance determined in the crude GMP and its fractions obtained by anion exchange chromatography (Figure 1).

Figure 1: Anion exchange chromatography of the crude GMP on a 1.5 × 5.7 cm column of DEAE-Sephacel. (A): Fractions collected were monitored for UV absorbance and sialic acid. (B): Fractions collected were also monitored for galactose, galactosamine, and phosphorus. Horizontal bars denote the fractions that were pooled for further study.
In addition to the major peak, the purified GMP also had a small peak at ~43 min (Figure 2A), which is likely related to the small peak that eluted near the total column volume on Sephacryl S-100 chromatography (see below).

The purified GMP was further studied using cellulose acetate electrophoresis and gel filtration chromatography on Sephacryl S-100. Cellulose acetate electrophoresis (Figure 3) showed a single but relatively broad peak of GMP sialic acid in fractions 8–10, which were seen to contain phosphorus, confirming that the purified product contained sialylated phosphorylated glycopeptide as shown above by DEAE-Sephacel chromatography (Figure 1).

Elution patterns of the purified GMP on Sephacryl S-100 gel filtration chromatography are given in Figure 4. With 0.05 M sodium phosphate-0.15 M NaCl, pH 7.0 as an eluent, greater than 99.5% of the purified GMP, monitored by sialic acid assay and UV absorbance measurement, eluted as a single peak with its elution volume close to the elution volume of dimeric β-lactoglobulin (36.6 kDa) (Figure 4A). A small peak corresponding to <0.5% of total recovered sialic acid or peptide appeared near the total column volume (33.0 ml determined using NaCl), which might have been a mixture of degradation products of GMP. This was not investigated further. In contrast to the results obtained above, when chromatographed with 6 M guanidine hydrochloride-0.1 M sodium acetate, pH 7.0, as an eluent, the GMP eluted at the position close to the elution position of α-lactalbumin (14.2 kDa) (Figure 4B). This indicated that the purified GMP was in aggregated form with 0.05 M sodium phosphate-0.15 M NaCl, but in disaggregated form in the presence of 6 M guanidine hydrochloride (dissociating agent). These results confirm the finding of Nakano and Ozimek [29] who reported that GMP aggregate is comprised of approximately three monomers.

In this study, most of non-sialylated GMP (not quantified in this study) or low-sialylated GMP was unlikely adsorbed to the anion exchanger, and thus not included in the purified GMP. This caused the apparently low recovery of purified GMP (average 43%). However, for preparation of foods for PKU patients, the level of phenylalanine in GMP is very important. We need a reliable method for constant supply of GMP with undetectable level of phenylalanine, which helps dietitians to estimate GMP content to be safely used in foods for PKU patients. The purified GMP can also be used as a research chemical. It is interesting to know whether the laboratory scale technique in this study can be scaled up (preferably with batch method) without losing its high reproducibility.

No attempt was made in this study to further separate impurities from the non-sialylated and low-sialylated GMP. Since the isoelectric point (pI) of GMP peptide (calculated to be 4.04 and 4.14 for its genetic variants A and B, respectively [11]) is close to the pI of α-lactalbumin (4.2–4.5) [1], it is uncertain whether ion exchange method is an efficient technique for selective separation of phenylalanine containing impurities from non-sialylated or low-sialylated GMP. Further research is needed to develop inexpensive methods to separate non-sialylated or low-sialylated GMP from sweet whey proteins.

### Table 2: Concentrations (g/100g) of carbohydrate and phosphorus in the crude GMP and products obtained by anion exchange chromatography.

|          | Crude GMP | Product adsorbed on the column (purified GMP) | Product unadsorbed on the column |
|----------|-----------|---------------------------------------------|----------------------------------|
| Sialic acid | 8.44 ± 0.47 | 15.51 ± 1.75 | 0.82 ± 0.47 |
| Galactose | 3.25 | 5.10 ± 0.85 | 0.70 ± 0.11 |
| Galactosamine | 3.85 | 6.14 ± 1.74 | 0.94 ± 0.26 |
| Phosphorus | 0.42 | 0.43 ± 0.02 | 0.62 ± 0.05 |

*Average of two determinations.
*Mean ± SD (n = 6).

Figure 3: Electrophoresis of the purified GMP on a cellulose acetate strip in 0.1 M pyridine-1.2 M acetic acid, pH 3.5.

Figure 2: Size exclusion HPLC on Superdex-75. (A): Elution patterns for the crude GMP, purified GMP, and unadsorbed product are shown. The purified GMP had a small peak eluting at ~43 min shown by an arrow. (B): The chromatogram shows elution patterns of molecular size markers including: (1) bovine serum albumin (67 kDa); (2) dimeric β-lactoglobulin (36.6 kDa); (3) cytochrome-C (13.6 kDa); (4) aprotinin (6.512 kDa); and (5) vitamin B12 (1.355 kDa).

Figure 4: Elution patterns of the purified GMP on Sephacryl S-100 gel filtration chromatography are given. With 0.05 M sodium phosphate-0.15 M NaCl, pH 7.0 as an eluent, greater than 99.5% of the purified GMP, monitored by sialic acid assay and UV absorbance measurement, eluted as a single peak with its elution volume close to the elution volume of dimeric β-lactoglobulin (36.6 kDa) (Figure 4A). A small peak corresponding to <0.5% of total recovered sialic acid or peptide appeared near the total column volume (33.0 ml determined using NaCl), which might have been a mixture of degradation products of GMP. This was not investigated further. In contrast to the results obtained above, when chromatographed with 6 M guanidine hydrochloride-0.1 M sodium acetate, pH 7.0, as an eluent, the GMP eluted at the position close to the elution position of α-lactalbumin (14.2 kDa) (Figure 4B). This indicated that the purified GMP was in aggregated form with 0.05 M sodium phosphate-0.15 M NaCl, but in disaggregated form in the presence of 6 M guanidine hydrochloride (dissociating agent). These results confirm the finding of Nakano and Ozimek [29] who reported that GMP aggregate is comprised of approximately three monomers.
chromatography is a relatively simple reproducible method to selectively remove phenylalanine impurities from crude GMP. The purified product is a GMP with high sialic acid content. To our knowledge, this is the first report of food grade GMP preparation without detectable level of phenylalanine. Further research is needed to scale up the method for industrial production of GMP as a food for PKU patients. It is also important to develop methods to recover non-sialylated and low-sialylated GMP which were removed with phenylalanine impurities during anion exchange chromatography, and thus not included in the purified GMP fraction in the present study.

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