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# Use of batch anion exchange technique for separation of κ-casein glycomacropeptide from bovine whey fraction

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## Research Article

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**Abstract**

Bovine κ-casein glycomacropeptide (GMP) is a sialic acid containing glycopeptide, which is considered as a health promoting compound found in cheese whey. The study described in this research communication was undertaken to determine whether GMP with undetectable level of contaminating protein or phenylalanine can be isolated from bovine whey fraction using batch anion exchange technique with chitin as an adsorbent. A soluble whey fraction (SWF) prepared from 1 g whey protein isolate (WPI) was mixed with a slurry of 1 g chitin, and the mixture was incubated at pH 3.0. After incubation, the mixture was filtered, and the residue obtained (containing chitin-GMP complex) was washed with water and eluted stepwise with 0.5 M NaCl and 2.0 M NaCl. Most of GMP (corresponding to 75.8% of total sialic acid recovered) was eluted with 0.5 M NaCl. The recovered GMP accounted for 5.4% dry weight of WPI (or 18.9% dry weight of SWF). Amino acid analysis showed that there was no detectable level of contaminating amino acids including phenylalanine, histidine, arginine and tyrosine in the GMP fraction. It was concluded that the batch anion exchange method with chitin developed in this study can be used for the isolation of high purity GMP from bovine SWF.

Bovine glycomacropeptide (GMP) is a bioactive compound found in cheese whey (Brody, 2000; Córdova-Dávalos *et al.*, 2019; Feeney *et al.*, 2019), which is considered to be useful as an ingredient for functional foods and pharmaceuticals. It is a sialylated phosphorylated glycopeptide released from κ-casein by the action of chymosin, a proteinase which catalyzes the cleavage between residues 105 (phenylalanine) and 106 (methionine) of bovine κ-casein during cheese manufacturing (Eigel *et al.*, 1984). GMP, which does not contain aromatic amino acids including phenylalanine, is also considered to be suitable as an amino acid source for the diet of patients suffering from phenylketonuria (PKU), a hereditary disorder of phenylalanine metabolism (see Pena *et al.*, 2018 for review). In PKU patients having no phenylalanine hydroxylase (an enzyme catalyzing the conversion of phenylalanine to tyrosine), phenylalanine from food protein, if ingested, accumulates in the body causing mental retardation. In order to prepare GMP which is safe for use in the diet of PKU patients, it is important to develop a method to isolate GMP with phenylalanine or protein levels that are as low as possible.

Anion exchange chromatography is one of the methods used to selectively separate GMP from whey proteins (Abd Ed-Salam, 2006; Feeney *et al.*, 2019). Nakano and Ozimek (2015) refined commercially available preparation of GMP by removing phenylalanine impurities using anion exchange chromatography on diethylaminoethyl (DEAE)-Sephacel. More recently, Nakano *et al.* (2018) deproteinized whey protein suspension by heat and acid treatments to obtain soluble whey fraction (SWF). They chromatographed SWF on DEAE-Sephacel to isolate GMP with undetectable level of contaminating amino acids including phenylalanine. These authors suggested removal of whey protein is important for efficient separation of GMP. Nakano and Betti (2020) then tested food grade anion exchange resins (e.g. Dowex 1 × 2, Diaion HPA75) and shrimp shell chitin (used as anion exchange resin) for column chromatographic separation of GMP from SWF. They reported that the amino acid composition of GMP eluted from each column was similar to that reported for GMP purified by DEAE-Sephacel chromatography (Nakano *et al.*, 2018).

For large industrial scale production of GMP, fractionation of whey using batch anion exchange method may be more convenient and economical compared to column chromatographic method, if it works well. There is, however, limited information available concerning the use of batch method for isolation of GMP. Ayers *et al.* (1998) fractionated whey protein concentrate using a batch technique with QA GiboCel anion exchanger, and separated GMP with low phenylalanine content (0.5% of total amino acids). This study was undertaken to determine whether GMP with undetectable level of phenylalanine is separated from SWF



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using batch anion exchange method with chitin extracted from shrimp shell. Chitin was used due to its potential for low cost food grade anion exchanger.

## Materials and methods

### Materials

A sample of 90% whey protein concentrate (WPI) was obtained from Vitarus Nutrition Inc., Abottsford, BC, Canada. Chitin used as anion exchange resin was the product prepared from shrimp shell in our previous study (Nakano and Betti, 2020). Approximately 8% of this preparation was glucosamine, whose primary amino group ($NH_2$) has a potential to bind negatively charged GMP. Sialic acid (*N*-acetylneuraminic acid) from *Escherichia coli* and 2-thiobarbituric acid were obtained from Sigma-Aldrich Canada Ltd., Mississauga, ON, Canada.

GMP standard was the product prepared by us (Nakano and Ozimek, 2015) by refining the commercial GMP by anion exchange chromatography. All solutions were prepared using deionized water further purified through a Milli-Q system (Millipore Ltd.).

### Isolation of GMP from WPI

To a sample of 1 g dry WPI powder, 20 ml water was added, and the mixture was subjected to heat treatment (98.5°C, 10 min) and then acid treatment with pH shift to 4.6 as described previously (Nakano *et al.*, 2018; Nakano and Betti, 2020) to obtain SWF. The SWF was adjusted to pH 3.0 with 1 M HCl and mixed with slurry of chitin (1 g) equilibrated in water (pH 3.0). The mixture was brought to 50 ml with water (pH 3.0), and incubated on a rotating mixer (Barnstead International, IA, USA) at 4°C for 20 h. After incubation, the mixture was filtered through filter paper (Q8 coarse filter paper, Fisher Scientific, Edmonton, AB, Canada). The residue collected on filter paper was washed with water (pH 3.0), and then eluted stepwise with 0.5 M NaCl and 2.0 M NaCl. Fractions collected (50 ml and 5–10 ml for components unadsorbed and adsorbed to chitin, respectively) were monitored for UV absorbance at 220 nm and sialic acid. Fractions containing sialic acid, eluting with 0.5 M NaCl, were pooled, dialyzed in water, freeze-dried and stored (as a GMP fraction) at 4°C until analyzed. This experiment was carried out in triplicate.

### Analytical methods

Sialic acid contents in WPI, SWF and fractions obtained during fractionation of SWF were determined as described previously using *N*-acetylneuraminic acid as a standard sialic acid (Nakano *et al.*, 2007). Amino acid compositions of SWF and GMP fraction were determined on samples hydrolyzed in glass distilled 6 M HCl in the presence of nitrogen gas as previously described (Nakano *et al.*, 2018).

## Results and discussion

### Separation of GMP from SWF with batch anion exchange method with chitin

The SWF recovered after removal of protein precipitates from WPI suspension corresponded to an average 28.5 ± 0.4% dry weight of WPI. This is close to the proportion of SWF (29.5 ± 0.9%) recovered from WPI in our previous experiment (Nakano and Betti, 2020). Sialic acid recovered in SWF accounted for 85.9 ± 3.3% of total sialic acid in WPI.

Figure 1 shows a fractionation pattern of SWF by batch anion exchange method with chitin. A minor proportion (22.6 ± 0.5%) of recovered sialic acid failed to bind to chitin, while most (75.8 ± 0.3%) of recovered sialic acid bound to chitin and eluted with 0.5 M NaCl. Elution was continued with 2.0 M NaCl and a small proportion of total recovered sialic acid (1.6 ± 0.3%) was released. These results are comparable to those reported previously (Nakano and Betti, 2020), which showed that 25% of total recovered sialic acid is not adsorbed on chitin column and the remaining 75% is adsorbed and eluted with 0.2–0.6 M NaCl gradient. The 0.5 M NaCl eluates were pooled to obtain a GMP fraction, which was subjected to further analysis, while the fraction containing unbound component and the 2.0 M NaCl fraction were not studied further.

Analytical data for the GMP fraction are given in Table 1, where for the purpose of comparison, analytical data for WPI, SWF and GMP purified by chromatographic method with chitin column (Nakano and Betti, 2020) are also presented. The recovered GMP accounted for 5.4% dry weight of WPI (or 18.9% dry weight of SWF calculated from its recovery percent shown in Table 1). The recovery of GMP will be increased by increasing the pH value for incubation mixture of SWF and chitin, but at the same time, the chance for contaminants to be adsorbed on chitin will be increased. For the production of GMP which is

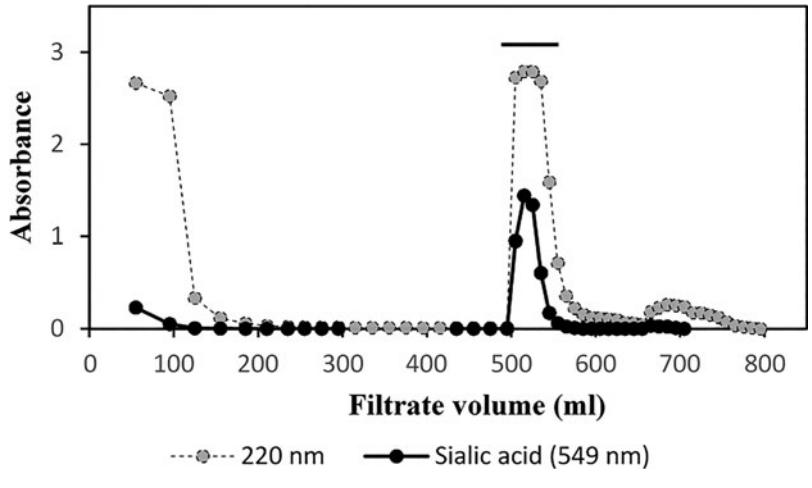


**Fig. 1.** Representative diagram showing fractionation pattern of SWF by batch anion exchange method with shrimp chitin as an adsorbent. The mixture of SWF and chitin incubated as described in the text was filtered to obtain the filtrate (0–55 ml) containing component unadsorbed on chitin. The residue left on the filter paper containing GMP-chitin complex was first washed with water (pH 3.0) (~ 440 ml), and then with 0.5 M NaCl (filtrate volume 495–570 ml) to elute most GMP. Further wash was carried out with 2.0 M NaCl (~140 ml). Fractions (5–50 ml) collected were first monitored for UV absorbance at 220 nm, and then an aliquot (0.02–0.1 ml) of each fraction was determined for sialic acid. A horizontal bar denotes eluates pooled, dialyzed in water and freeze-dried to obtain the GMP fraction for further study. See the text for other details.

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**Table 1.** Analysis of WPI, SWF and GMP fractions prepared by batch anion exchange method with chitin and by chromatography on chitin column

| | WPI | SWF | GMP (batch method) | GMP[a] (chromatography) | GMP[b] $V_A$ | GMP $V_B$ |
|---|---|---|---|---|---|---|
| Recovery (% dry weight of WPI) | | 28.5 ± 0.4[c] | 5.4 ± 0.3 | 5.3 ± 0.1 | | |
| Sialic acid (% dry weight) | 1.5 ± 0.1 | 4.1 ± 0.3 | 12.6 ± 0.3 | 13.1 ± 0.4 | | |
| Amino acid[d] (mol%) | | | | | | |
| Asx | 11.2[e] | 9.3 ± 0.2 | 7.4 ± 0.4 | 7.7 ± 0.2 | 7.8 | 6.3 |
| Ser | 7.6 | 10.1 ± 0.1 | 11.1 ± 0.3 | 9.9 ± 0.8 | 9.4 | 9.4 |
| Glx | 15.5 | 16.8 ± 0.1 | 15.9 ± 0.4 | 16.4 ± 0.2 | 15.6 | 15.6 |
| Gly | 3.1 | 2.3 ± 0.0 | 2.2 ± 0.2 | 2.2 ± 0.1 | 1.6 | 1.6 |
| His | 1.6 | 0.8 ± 0.0 | nd | nd | 0 | 0 |
| Arg | 2.2 | 1.0 ± 0.1 | nd | nd | 0 | 0 |
| Thr | 8.4 | 13.8 ± 0.2 | 17.2 ± 0.3 | 18.2 ± 0.4 | 18.8 | 17.2 |
| Ala | 7.0 | 6.4 ± 0.1 | 7.3 ± 0.1 | 7.9 ± 0.1 | 7.8 | 9.4 |
| Pro | 6.6 | 10.6 ± 0.1 | 12.5 ± 0.6 | 11.9 ± 0.2 | 12.5 | 12.5 |
| Tyr | 2.1 | 0.8 ± 0.0 | nd | nd | 0 | 0 |
| Val | 6.8 | 7.8 ± 0.1 | 9.4 ± 0.3 | 9.3 ± 0.4 | 9.4 | 9.4 |
| Lys | 8.5 | 6.0 ± 0.1 | 4.8 ± 0.2 | 4.6 ± 0.1 | 4.7 | 4.7 |
| Ile | 6.3 | 8.3 ± 0.1 | 9.8 ± 0.3 | 10.1 ± 0.6 | 9.4 | 10.9 |
| Leu | 10.4 | 4.7 ± 0.1 | 2.5 ± 0.3 | 2.1 ± 0.1 | 1.6 | 1.6 |
| Phe | 2.6 | 1.3 ± 0.1 | nd | nd | 0 | 0 |

nd, Not detected.
[a]GMP purified by chromatography on chitin column (Nakano and Betti, 2020).
[b]Amino acid composition of genetic variant A ($V_A$) or variant B ($V_B$) is based on its primary structure (Eigel *et al.*, 1984).
[c]All data with the exception of amino acid analysis data for WPI are expressed as mean ± SD ($n = 3$).
[d]Cysteine, methionine and tryptophan were not determined.
[e]Analyzed by Nakano *et al.*, (2018: see Supplementary Table S2 of this reference).

safe as food for PKU patients, the purity of GMP (determined by phenylalanine assay) is more important than its yield. All results including the recovery of GMP, sialic acid concentration and amino acid composition were comparable between GMP prepared by the batch method and that prepared by the chromatographic method. The sialic acid content was approximately three and eight times higher in SWF and the GMP fraction, respectively than in WPI. This indicated that during purification of GMP, its purity was increased eight-fold in the GMP fraction by eliminating protein precipitates from WPI followed by selective adsorption of GMP from SWF on chitin anion exchanger. This is consistent with changes in amino acid composition, in that the content of phenylalanine, histidine, arginine or tyrosine (each of which is amino acid absent in GMP) was reduced in SWF to ≤ 50% of the content of amino acid in WPI, and not detectable in the final product of GMP fraction. This indicated that the batch anion exchange method with chitin can be used for isolation of high purity GMP which may be suitable for the diet of PKU patients.

Although not examined in this study, it is of interest to investigate the presence of bioactive compounds other than GMP, if any, in the SWF components which were not adsorbed on chitin (Fig. 1). This approach makes sense since Sommella *et al.* (2016) analyzed peptides from buffalo's scotta, a liquid by-product containing SWF obtained by ricotta cheese production from buffalo's whey. These authors reported the presence of bioactive compounds (e.g. those with antihypertensive, antimicrobial, and antioxidant activities) in their peptide fraction.

In conclusion, the present results suggest that, firstly, the SWF with relatively low protein content is an important material for isolation of GMP with anion exchange technique and, secondly, the batch anion exchange method with chitin is a relatively simple and inexpensive technique to selectively separate GMP from SWF with undetectable level of protein or phenylalanine impurities. It appears that the method can be scaled up without difficulty.

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