International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
www.chemijournal.com
IJCS 2020; SP-8(6): 246-251

Received: 09-08-2020
Accepted: 16-09-2020

Pushpa BP
Department of Dairy Chemistry, Dairy Science College, KVAFSU, Bengaluru, Karnataka, India

Jayaprakasha HM
Department of Dairy Technology, Dairy Science College, KVAFSU, Bengaluru, Karnataka, India

Rathnamma D
Department of Veterinary Microbiology, Veterinary College, KVAFSU, Bengaluru, Karnataka, India

Corresponding Author:
Pushpa BP
Department of Dairy Chemistry, Dairy Science College, KVAFSU, Bengaluru, Karnataka, India

Characterization of glycomacropeptides (GMP) for its physico-chemical properties obtained from cow and buffalo casein hydrolyzates

Pushpa BP, Jayaprakasha HM and Rathnamma D

DOI: https://doi.org/10.22271/chemi.2020.v8.i6d.11162

Abstract
The objective of the study was to characterize the glycomacropeptides (GMP) for its physico-chemical properties (glycosylation, absorbance pattern in UV region, MW profile and RP-HPLC profile) obtained from cow and buffalo casein hydrolyzates. Higher sialic acid content was observed in buffalo caseinate GMP (1.35%) as compared to cow GMP (1.20%). The absorption (OD) pattern in the UV region demonstrated that GMP measurement between 220 and 250 nm is suitable range. Peptide profile on SDS PAGE based on the MW and RP-HPLC has confirmed the presence of GMP along with other minor peptides as impurities for both cow and buffalo caseinate GMP. From the study it can be concluded that GMP obtained from alcohol precipitation method may contain peptide impurities and require further purification for its best utilization in other food products.

Keywords: Glycomacropeptides, sialic acid, UV pattern, SDS-PAGE, RP-HPLC

Introduction
Glycomacropeptides (GMP) are bioactive peptides having 64 amino acid residues, which are found in rennet cheese whey. They are the C-terminal part (f 106–169) of kappa-casein, released in whey during cheese making by the action of chymosin. GMP constitutes about 20–25% of total proteins in whey products like whey powder, whey protein isolates (WPI), whey protein concentrates (WPC), manufactured from cheese whey (Farias *et al.*, 2010; Robitaille, *et al.*, 2012) [1, 2].

GMP contain a unique amino acid profile which is significantly rich in the Proline, Glutamic acid, Serine and Threonine but depleted in aromatic amino acids such as Trptophane, Tyrosine, Phenyl alanine and Cysteine as revealed by Eigel *et al.* (1984) [3] based on the primary structure. The heterogeneous carbohydrate chains are the unique characteristics of GMP, not found in the remaining part (1 to 105) of κ -CN. Each of the GMP fractions with different carbohydrate chains might be responsible for different biological functions (Li and Mine 2004) [4]. The 3D structure of GMP through protein modelling demonstrated by Kreub *et al.* (2009) [5] showed that a large part of the peptide has a strong negative charge, while there are three small positively charged domains at the N terminus. Presence of sialic acid (glycan) in GMP is a special feature as compared to other milk derived peptides. Hence, GMP exhibits a different behaviour in terms of the physico-chemical properties as compared to other sources of proteins or peptides. Mercier and Chobert (1976) [6] illustrated that 16% variation in the buffalo GMP and cow GMP variant A, based on the primary structure.

In the current years, several biological functions of GMP have been exploited *viz*. binding of enterotoxins, inhibit bacterial and viral adhesions, modulation in immune system and promoting growth of probiotic cultures (Brody, 2000; Thoma-Worringer *et al*., 2006) [7, 8]. The unique amino acid profile and techno-functional along with biological properties encouraged its application as an ingredient for nutrition management, infant formulations, therapeutic and neutraceutical foods (Nakajima *et al*, 2005; LaClair *et al*., 2009) [9, 10]. Hence, researchers have developed a number of protocols for isolation of GMP from cheese whey has been reported. Saito *et al*. (1991) [11] and Berrocal and Neeser (1993) [12] isolated GMP from sweet cheese whey by alcohol precipitation and ion exchange chromatography after heat coagulation of whey protein.

Li and Mine (2004) [4] isolated GMP from WPI by TCA to study chromatographic profile whereas Fukuda *et al*. (2004) [13] used 12% TCA to precipitate GMP in milk. Different commercial methods have been described for the isolation of GMP by means of ultrafiltration (Minkiewicz *et al.*, 1996; Nakano and Ozimek, 2002) [14, 15] based on differences in the MW; using micro filtration (MF) and dia filtration (DF). Thoma-Worringer and Kulozik (2004) [16] concentrated the whey permeate containing GMP using UF membrane and detected GMP by RP –HPLC. Several research carried out to purify GMP using cation exchange chromatography, ultrafiltration / diafiltration, and lyophilization at the pilot plant scale to decrease Phenylalanine for Phenylketonuria patients (LaClair *et al*., 2009) [10]. Nakano and Ozimek (2000) [17] purified GMP from chymosin-hydrolyzed caseinate solution by the procedure involving gel chromatography on Sephacryl S-200 at pH 7.0.

The present study focuses on to produce GMP at laboratory level, sodium caseinate from cow and buffalo skim milk were used as a starting material so that GMP would contain less residual impurities of fat, lactose and whey proteins. As on now, there is no literature available regarding isolation of GMP from buffalo milk caseins and their characterization. GMP was produced by hydrolyzing cow and buffalo sodium caseinates using chymosin enzyme and extracted by ethyl alcohol precipitation method to characterize for its physico-chemical properties.

Materials and methods

Whole cow milk from Dairy Farm, Veterinary College, Bengaluru and buffalo milk procured from local dairy farms was used for the study.

Skim milk preparation: Cow and buffalo milk were subjected to centrifugation (Ultracentrifuge; JOURAN BR4) under cold conditions (10°C) at 3000 rpm for 10 min. Skim milk was separated by pouring into the container carefully, by piercing the top cream layer in the tube.

Sodium caseinate preparation: Acid casein (wet) was prepared by adjusting the pH of skim milk to 4.6 with 10% HCl at 38°C, casein and whey were separated by filtration. Casein was washed with acidic chilled water (pH 4.0, 10°C) two times and then with normal deionized water (pH 7.0) at room temperature. Sodium caseinate was prepared by dissolving acid casein in 0.5% NaOH solution at 65°C by adjusting the pH to 7.0 and freeze dried.

Chymosin enzyme: Microbial source Chymosin (rennet) enzyme with activity of 2 g per 100 kg of cheese milk was obtained from Chr. Hansen Laboratories, Copenhagen, Denmark.

Protein Molecular Weight Marker (Low Range): For SDS Gel Electrophoresis with a MW range of 3,500-45,000 D was purchased from Aristogene Biosciences *Pvt. Ltd*., Bengaluru.

Standard Glycomacropeptide (GMP): Commercial spray dried GMP (90% purity) isolated from cheese whey, a kind gift from AGROPUR Ingredients, Minnesota.

Production of GMP: GMP was produced in the laboratory by hydrolysing the sodium caseinate using chymosin enzyme and precipitating by ethyl alcohol by adopting the method followed by Pushpa *et al*., 2018 [18].

Sialic acid content analysis: Sialic acid content reflects the extent of glycosylation, was estimated by the acidic ninhydrin method (Fukuda *et al*., 2004) [13]. Colour development was measured at 470 nm and concentration (µg /ml) of the unknown sample (GMP) was read on sialic acid standard curve as delineated in the Fig.1.

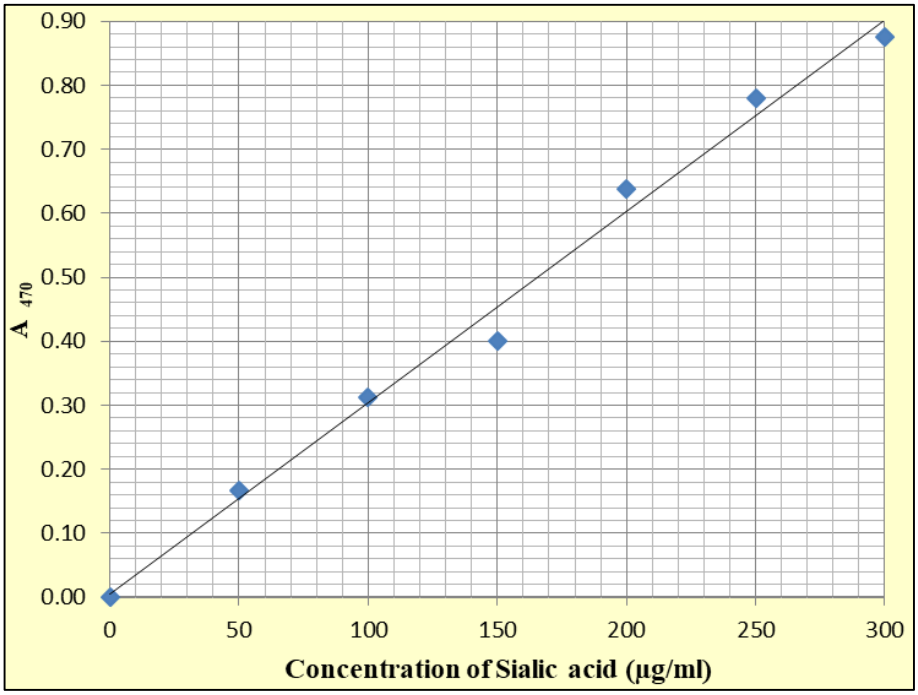


Fig 1: Standard curve for determination of sialic acid content

UV Characterization

GMP solutions (10-100 µg / ml) were prepared in 0.1 M phosphate buffer solution (pH -7.0) and filtered through 0.45 µm pore size filter. The absorbance of GMP solution (0.05 mg/ml; w/v) at different wavelengths between 205-280nm were measured using UV–VIS spectrophotometer

(SECOMAM). The absorbance values recorded were plotted against wavelength and maximum absorbance was noted for particular GMP dilution.

Molecular Weight analysis

SDS –PAGE was carried out to check the purity of GMP obtained from cow and buffalo skim milk casein hydrolysates. Gels with 4 and 15% w/v acrylamide were prepared for stacking and resolving respectively were prepared. Mini Protean III BIO-RAD electrophoretic apparatus (Bio –Rad Pacific Limited, Quarry Bay, Hongkong) was used to carry out the electrophoresis according to the procedure given by Sambrook *et al*. (2012) [19] with slight modification.

RP HPLC analysis

RP-HPLC was performed for GMP from cow and buffalo casein to its purity and to characterize peptides. GMP were analysed by UFLC (Ultra flow Liquid chromatography) system (SHIMADZU, Japan) by adapting the procedure followed by Molle and Leonil (2005) [20]. RP-HPLC system equipped with a prime sphere C-18 column (Luna® omega, 3 µm PSC 18, 100 A^0) fitted with guard column (3.2 X 30 mm). The system consists of a CTO-10ASVP a system controller, SIC-10 A auto injector, a SPD-M 20 A detector (PDA-photo diode array) and two LC-20 AD pumps, all integrated with a data manipulation system. GMP samples of 10 mg from cow and buffalo caseinate and standard sample were dissolved in 10 ml of HPLC water along with 40 µ l of TFA. The solution was then filtered through 0.22 µ m sterile syringe filters (AXIVA); 2.5 mm the filtrate was collected in a clean dry test tube. The solvent A (0.106% (v/v) Trifluoroacetic acid -TFA) and solvent B (0.1% (v/v) TFA in acetonitrile HPLC water; 80:20) was prepared in HPLC grade water. The column was initially equilibrated with 20% of solvent B. Samples were applied to the column and eluted by a linear gradient of solvent B performed as follows: 0-3 min, 20%; 3-25 min, 20-55%; 25-28 min, 55-80%; then the column was held at 80% for 3 min. The flow rate was set to 1 ml / min for detection time for 30 min at 280 nm absorbance. The graph obtained was analysed for number on peaks, retention time and area of the peak comparing with standard GMP.

Statistical analysis: The results are the average of three or five replications and were statistically analyzed by subjecting to statistical analysis using R Programme, R - Version 3.4.3.

Results and discussion

Sialic acid content in GMP

The results of sialic acid content in GMP obtained from cow and buffalo caseinate showed a significant difference ($P \leq 0.05$) with higher sialic acid content in buffalo GMP (1.35%,w/w) as compared to cow GMP (1.20%). However, the sialic content in both the samples were very less when compared to pure form of GMP isolated and purified by several workers by adopting advanced methods of isolation and purification. Method of extraction has an influence on the glycosylation of GMP as studied by Li and Mine (2004) [4], who isolated GMP by alcohol precipitation method has shown GMP with 75.70% glycosylation as compared to UF separation (81.60%). On the contrary, Nakona and Ozimek (2002) [15] and Laclair *et al*. (2009) [10] noted that GMP obtained from cheese whey by adopting membrane filtration and ion exchange chromatography resulted with sialic acid content of more than 10% while, Fernando and Woonton (2010) [21] obtained sialic acid content in the range of 5-11.3% in GMP isolated from cow cheese whey by liquid chromatography.

UV absorbance pattern of GMP

The absorbance readings taken in the UV range (205-280 nm) for different sources of protein such as WPC, cow and buffalo caseinate, GMP from cow and buffalo caseinate are depicted in the Table 1. The absorbance values at 205 nm were observed to be higher > 1.5 OD for all protein sources except WPC (< 1.0 OD). The values at 205 nm were 0.630, 1.742, 2.022, 1.637 and 2.040 for WPC, cow casein, buffalo casein, GMP from cow casein and GMP from buffalo casein, respectively as compared to absorbance at 280 nm was 0.052, 0.086, 0.077, 0.037 and 0.035, respectively for the above samples. However, the extent of difference in absorption between 220 and 250 nm (< 1.0 OD) was found to be non-significant indicating the suitable range of wavelength which can be used for measuring the absorbance values for GMP. A significant difference between the absorbance values of different protein sources and UV wavelengths were observed. The results of the present study was in agreement with the Oliva *et al*. (2002) [22] who revealed that 205-226 nm was ideal for detection of GMP and differences in 210 and 280 nm were frequently used to characterize GMP in HPLC studies. Molle and Leonil (2005) [20] and Bonnaille *et al*. (2014) [23] have characterized GMP for non- glycosylated and glycosylated peptides by RP-HPLC method at 214 nm while Thoma-worringer *et al*. (2006) [8] taken readings in their work at 226 nm for GMP.

Table 1: Absorbance pattern of type of protein sources at UV region

Source of protein	UV range (nm)				
	205	220	250	280	CD ($P \leq 0.05$)
	Absorbance (OD)				
WPC	0.630^{bA}	0.360^{bB}	0.135^{bC}	0.052^{abC}	0.09
Cow caseinate	1.742^{aA}	0.935^{aB}	0.807^{aB}	0.086^{aC}	0.28
Buffalo caseinate	2.022^{aA}	1.062^{aB}	0.835^{aB}	0.077^{bC}	0.29
Cow caseinate GMP	1.637^{aA}	0.820^{aB}	0.642^{aB}	0.037^{abC}	0.35
Buffalo caseinate GMP	2.040^{aA}	0.995^{aB}	0.670^{aC}	0.035^{abD}	0.30
CD ($P \leq 0.05$)	0.29	0.29	0.37	0.30	

Note

- Average of 5 trails
- Similar superscripts indicate NS at the corresponding CD
- Superscripts: Small letter signifies the wavelength Capital letter signifies the sources of protein

Molecular weight profile of GMP

The molecular weight profile of GMP from cow and buffalo caseinates are delineated in Plate 1 against Protein Marker of MW range from 3,500 to 45,000 D. It is evident from the results that peptide band was observed slightly above the 6,500 D region for both cow and buffalo GMP indicating the presence of GMP in the sample, probably in monomer state. Two more major bands, one in the region between 14,300 and 18,400 D and other at 3,500 D region was observed in both cow and buffalo casein GMP samples. The presence of 2 or 3 peptides additional to GMP peptide indicates the low purity of sample and it may require some purification processes like chromatography techniques and ultra-filtration. The behaviour of GMP in aggregated form having higher MW peptides at different pH values was observed by many workers (Kawasaki *et al.*, 1993; Galindo-Amaya *et al*., 2006; Rojas and Torres, 2013) [24, 25, 26]. They also illustrated existence of GMP in dimers (14 kD), trimers (21 kD) or oligomers (upto

30 kD) by association of monomers, as a function of pH. The studies of Farias *et al*., (2010) [1] reported that GMP existed in associated forms (23 -28 kD) in alkaline pH and partial dissociation could be possible in acidic pH.

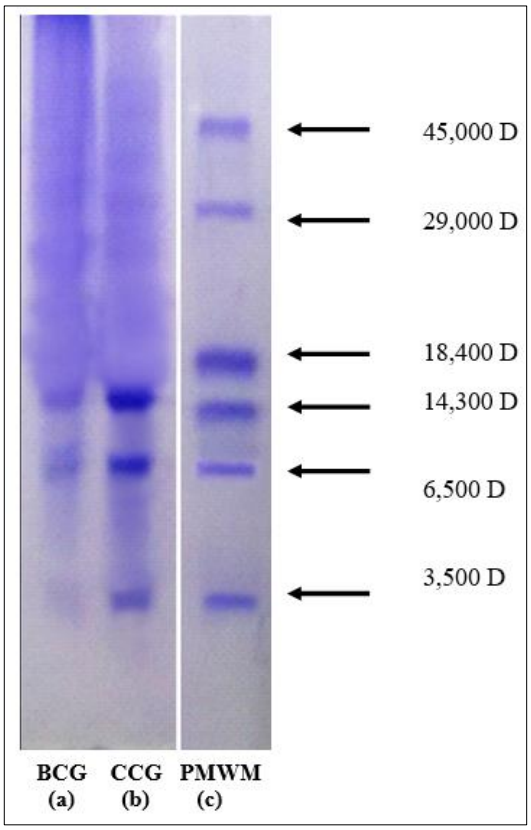


Plate 1: Molecular weight profile of Glycomacropeptides (GMP) by SDS-PAGE (a) BCG - Buffalo caseinate GMP (b) CCG - Cow caseinate GMP (c) PMWM – Protein Molecular Weight Marker

Characterization of GMP by RP-HPLC

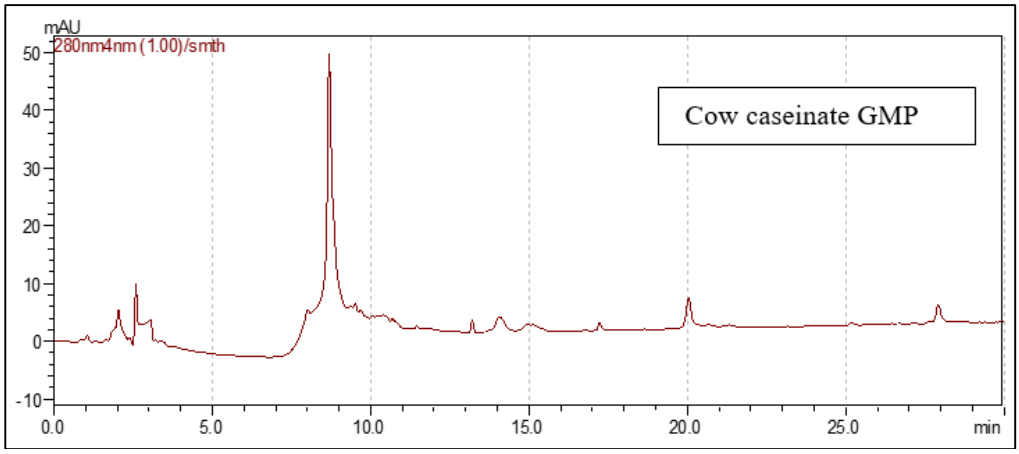
The RP-HPLC profile was carried out for the GMP obtained from cow and buffalo caseinate in comparison with the standard GMP with 90% purity. The peptides were characterized based on their relative retention time, area; height and concentration (% area) of the peak are presented in Table 2 and Fig. 2. It is evident from the results that the peak retention time of GMP from cow and buffalo caseinate was 8.67 and 8.63 min as against standard GMP (8.35 min). The peak retention time of the peptides is directly related to the polar nature of the peptides (*i.e.* hydrophilic peptides) and slightly higher retention time for cow and buffalo GMP was observed as compared to the standard GMP. Other 7-8 smaller peaks were observed between the retention time of 2-4 min, 12-14 min and 17 min in case of cow and buffalo GMP indicate the probable impurities. Table values revealed that the standard GMP showed higher values of peak area, height and concentration. The concentration in terms of area% for standard GMP, cow caseinate GMP and buffalo caseinate GMP were 23.42, 10.00 and 9.75, respectively. It can be concluded from the results that the cow and buffalo caseinate GMP were having lesser purity (44 and 42%, respectively) as compared to the standard GMP taking it as 100% purity for calculation purpose. Statistical analysis has shown a non-significant difference in the area, height and concentration between cow and buffalo caseinate GMP but, standard GMP has shown significant difference ($P \leq 0.05$) with other two samples. The results obtained in the present investigation are in line with the findings of Bonnaillie *et al.* (2014) [23] who reported that GMP was eluted from C-18 column between 3.7- 4 min with the peak area of 16.5 ($x10^{-6}$) for 31.7% purified GMP and 40.7 ($x10^{-6}$) for 58.7% purified GMP. Elution of GMP on C-18 column with a retention time of less than 10 min was reported by many workers (Liske *et al.*, 2004; Fukuda *et al*., 2004) [27,13].

Table 2: RP - HPLC profile pattern of different sources of Glycomacropeptides

Source of GMP	Retention time (min)	Area ($x 10^{-6}$)	Height ($x 10^{-5}$)	Concentration (Area %)	Purity (%)
Standard GMP	8.35	27.10^{a}	13.00^{a}	23.42^{a}	100
Cow caseinate GMP	8.67	11.90^{b}	10.00^{b}	10.00^{b}	44
Buffalo caseinate GMP	8.63	11.40^{b}	9.20^{b}	9.75^{b}	42
CD ($P \leq 0.05$)	-	0.89	0.04	0.26	-

Note:

- Standard GMP: AGROPUR Ingredients, Minnesota.
- Similar superscripts indicate NS at the corresponding CD



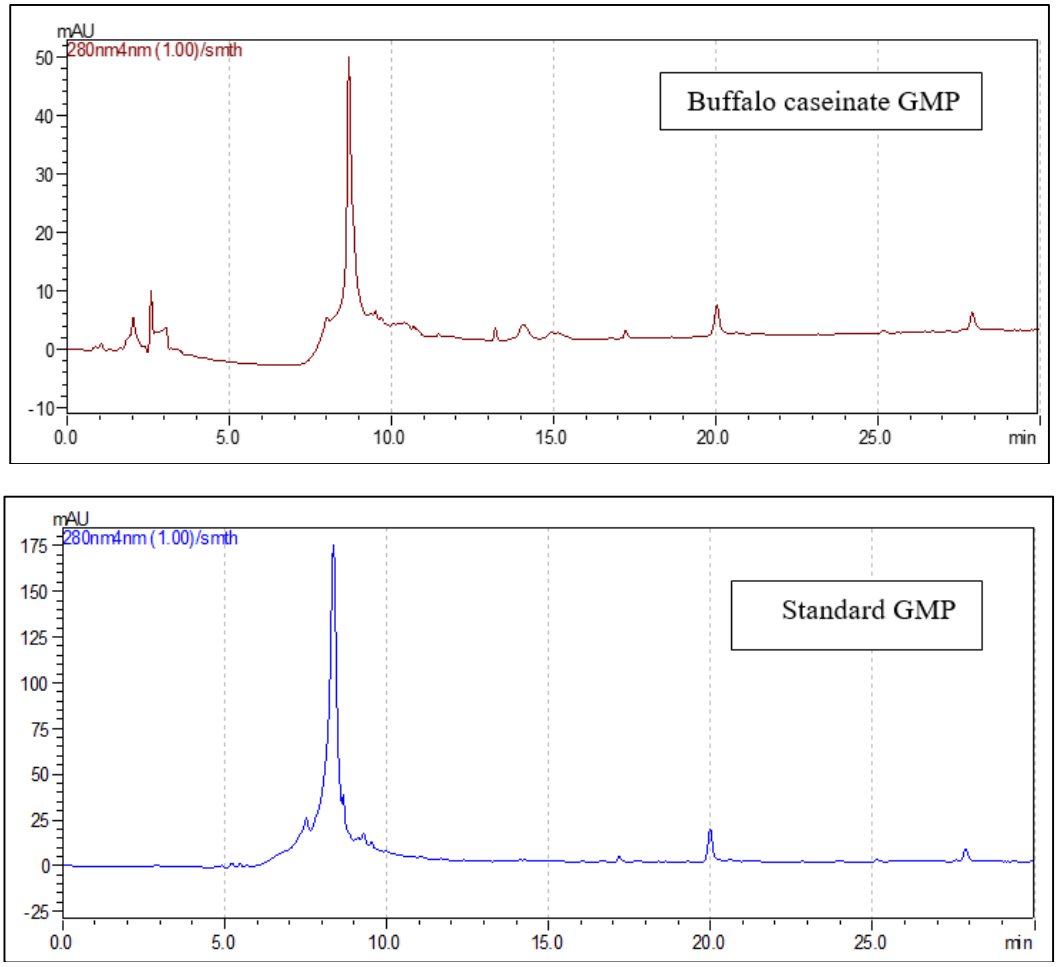


Fig 2: RP - HPLC profile pattern of different sources of glycomacropeptides (GMP)

Conclusion

The results of the study revealed that isolation of GMP by alcohol precipitation method had a greater influence on the extent of glycosylation (sialic acid content). RP-HPLC and SDS-PAGE indicated lower purity of GMP possessing other peptides of varying MW. Hence, advance purification or separation techniques must be adopted (chromatography and ultra-filtration) to obtain GMP with highest purity level.

Acknowledgements

We are grateful to Head of Biotechnology Department, GKVK, Bengaluru; Department of Veterinary Microbiology and Veterinary Biochemistry, Hebbal, Bengaluru for providing the necessary laboratory facilities.

References

1. Farías M, Martinez M and Pilosof A. Casein glycol macropeptide pH-dependent self assembly and cold gelation. International Dairy Journal 2010;20:79-88.
2. Robitaille G, Lapointe C, Leclerc D, Britten M. Effect of pepsin-treated bovine and goat caseinomacropeptide on *Escherichia coli and Lactobacillus rhamnosus* in acidic conditions. Journal of Dairy Science 2012;95:1-8.
3. Eigel W, Butler J, Ernstrom C, Farrell H, Harwalkar V, Jenness R *et al*. Nomenclature of proteins of cow's milk. Journal of Dairy Science 1984;67:1599-1631.
4. Li WY, Mine Y. Technical Note: Comparison of chromatographic profile of glycomacroepeptide from cheese whey isolated using different methods. Journal of Dairy Science 2004;87(1):174-177.
5. Kreub M, Thoma S, Kulozik U. The effect of glycosylation on the interfacial properties of bovine caseinomacropeptide. Food Hydrocolloids 2009b;23(7):1818-1826.
6. Mercier JC, Chobert JM. Comparative study of the amino acids sequences of the caseinomacropeptides from seven species. FEBS letters 1976;72(2):208-214.
7. Brody EP. Biological activities of bovine glycomacropeptide. British Journal of Nutrition 2000; 84(1):39-46.
8. Thoma-Worringer C, Krause I, Kulozik U. Precipitation behaviour of caseino macropeptides and their simultaneous determination with whey proteins by RP-HPLC. International Dairy Journal 2006;16:285-293.
9. Nakajima K, Tamura N, Kobayashi-Hattori K, Yoshida T, Hara-Kudo Y, Ikedo M et al. Prevention of intestinal infection by glycomacropeptide. Biosciences Biotechnology and Biochemistry 2005;69:2294-2301.
10. Laclair CE, Ney DM, Macleod EL, Etzel MR. Purification and use of glycomacropeptide for nutritional management of phenylketonuria. Journal of Food Science 2009;74(4):199-206.
11. Saito T, Yamaji A, Itoh T. A New Isolation Method of caseinoglycopeptide from sweet cheese whey. Journal of Dairy Science 1991;74(90):2831-2837.
12. Berrocal R, Neeser JR. Production of k-caseino-glycomacropeptide. US Patent 1993;5:216-129.
13. Fukuda SP, Roig SM, Prata LF. Correlation between acidic ninhydrin and HPLC methods to evaluate fraudulent addition of whey in milk. Dairy Science and Technology 2004;84:501-512.

14. Minkiewicz P, Slangen CJ, Lagerwerf FM, Haverkamp J, Rollema HS, Visser S. Reversed-phase high-performance liquid chromatographic separation of bovine k-casein macropeptide and characterization of isolated fractions. Journal of Chromatography A 1996;743:123-135.
15. Nakano T, Ozimek L. Isolation of glycomacropeptide from sweet whey by gel chromatography on sephacryl S-200 at pH 7.0 and 3.5. Milchwissenschaft 2002;57:128-130.
16. Thoma-Worringer C, Kulozik U. Methods of obtaining isolated caseinomacropeptide from milk and whey and functional properties. Bulletin IDF 2004;389:74-77.
17. Nakano T, Ozimek L. Purification of glycomacropeptide from dialyzed and non-dialyzed sweet whey by anion-exchange chromatography at different pH values. Biotechnology Letters 2000;22:1081-1086.
18. Pushpa BP, Jayaprakasha HM, Kempanna C, Jayashri P Hiremath, Arun Kumar. Production of glycomacropeptides (GMP) from cow and buffalo sodium caseinates. International Journal Trend in Research and Development 2018;5(4):423-427.
19. Sambrook J, Maccallum P, Russel D. Molecular cloning: A laboratory manual 2012; Edn. 4th Cold Springs Harbour Press, NY, ISBN 0-978-1936113-42-2.
20. Molle D, Leonil J. Quantitative determination of bovine κ-casein macropeptide in dairy products by liquid chromatography/electrospray coupled to mass spectrometry (LC-ESI/MS) and liquid chromatography/electrospray coupled to tandem mass spectrometry (LC-ESI/MS/MS). International Dairy Journal 2005;15(5):419-428.
21. Fernando SF, Woonton BW. Quantitation of *N*-acetylneuraminic (sialic) acid in bovine glycomacropeptide (GMP). Journal of Food Composition Analysis 2010;23(4):359-366.
22. Oliva Y, Escobar A, Ponce P. Caseinomacropéptido bovino: unaalternativapara la salud. Review Salud Animal 2002;24:73-81.
23. Bonnaille LM, Qi P, Wickham E, Tamosula PM. Enrichment and purification of casein glycomacropeptide from whey protein isolate using supercritical carbon dioxide processing and membrane ultrafiltration. Foods 2014;3(1):94-109.
24. Kawasaki Y, Kawakami H, Tanimoto M, Dosako S, Tomizawa A, Kotakem *et al*. pH dependent molecular-weight changes of kappa-casein glycomacropeptide and its preparation by ultrafiltration. Milchwissenschaft 1993b;48:191-196.
25. Galindo-Amaya LL, Vilbuena-Colmenares E, Rojas-Villarroel E. Standardization of glycomacropeptide detection with SDS-PAGE as a milk adulteration index. Revista Científica (Maracaibo) 2006;16:308-314.
26. Rojas E, Torres G. Isolation and recovery of glycomacropeptide from milk whey by means of thermal treatment. Food Science and Technology 2013;33(1):14-20.
27. Lieske B, Konrad G, Kleinschmidt T. Isolation of caseinomacropeptide from rennet whey by a multi-stage ultrafiltration process III. Influence of pH in the first ultrafiltration-step on the chemical properties of isolated caseinomacropeptide. Milchwissenschaft 2004;59(7, 8):408-410.