REVIEW: ISOLATION AND PURIFICATION OF MILK WHEY GLYCOMACROPEPTIDE

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The objective of this work was to review the isolation and purification methods of glycomacropeptide (GMP). This peptide is formed during enzymatic coagulation of milk, using chymosin. Aspects such as structure, composition, biological activities and functional and technological properties of GMP are also covered. It was concluded that the various methods mentioned in this paper for GMP isolation and purification use isolated or combined processes, which can be classified in three categories: selective precipitation induced by adjustment of the physical properties of the solution; membrane filtration based mainly on different membrane sizes and load, and selective adsorption. Regarding the biological activities of GMP, this peptide can be understood as a promising compound, although additional research is necessary in order to define quantities, efficacy and to allow functionality claims. There is, however, little information regarding GMP's addition in food, interaction with other components and chemical stability under different processing conditions.

KEY-WORDS: MILK WHEY; GLYCOMACROPEPTIDE; BIOLOGICAL ACTIVITY.

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1 INTRODUCTION

Milk whey can be obtained in laboratory or industry mainly by three processes: (a) enzymatic coagulation, resulting in casein coagulates, which are used for cheese and sweet whey production; (b) acid precipitation at the isoelectric point, resulting in isoelectric casein, which is transformed into caseinates and acid whey; and (c) physical separation of the casein micelle by microfiltration, originating a micelle concentrate and whey proteins, in the form of protein isolate or concentrate (SGARBIERI, 2004).

The amount of whey generated from cheese production depends on the type of cheese produced and on production process. For the production of one kg of cheese, 10 liters of milk are used and depending on the water, there is a whey production that varies from 9 to 12 liters (RICHARDS, 1997). Brazil ranks third place in world cheese producers, with 480 thousands of tons, behind only of the European Union and the United States (ANUÁRIO…, 2005).

Whey is not a polluting agent for itself, but when disposed in watercourses it causes great polluting effects (PORTO, SANTOS & MIRANDA, 2005). According to RICHARDS (2002), when whey is disposed without treatment it presents a high biological oxygen demand (BOD), of 30,000 to 50,000 mg of oxygen per liter of whey. This value is approximately 100 times more than domestic sewage. An industry with an average production of 10,000 liters of whey per day pollutes as much as a population of 5,000 inhabitants.

Because it is an abundant byproduct of cheese industries, milk whey has great economical interest as a protein source for the food industry. It offers a series of functional benefits in a number of applications, such as solubility, viscosity, emulsification, gelification, among others.

The main proteins in whey are α-lactalbumin, ß-lactoglobulin, immunoglobulin and glycomacropeptide (GMP). In lesser amounts, but with important commercial applications are lactoferrin and lactoperoxidase (DOULTANI, TURHAN & ETZEL, 2004).

GMP, found in sweet whey, is a biologically active compound liberated from k-casein by the action of chymosin on the peptidic bond Phe_{105}-Met_{106}, during cheese making. Due to its biological activity and potential as ingredient for functional foods, great attention has been paid to GMP isolation and purification. Since GMP has the amino acid residues 106-169, it contains no aromatic amino acids, so it may be used in foods for patient with phenylketonuria, an important innate error in phenylalanine metabolism (NAKANO et al., 2002; NAKANO and OZIMEK, 2000).

This work is a review of the GMP isolation and purification methods found in literature up to this moment. The search was for methods that used casein and calcium and sodium caseinates treated with chymosin and also cheese whey produced by enzymatic coagulation. The latter one is a very attractive option since it adds value to this dairy industry byproduct.

2 STRUCTURE AND COMPOSITION

GMP is a heterogeneous peptide of 64 amino acids formed by casein cleavage using chymosin during cheese production. The enzyme promotes rupture of the Phe_{105}-Met_{106} bond of k-casein, unstabilizing and denaturating the casein micelle, forming two peptides. One of the peptides, named para-k-casein contains the residues 1 to 105 and stays in the casein micelle. The other peptide is formed by the residues 106 to 169 and because it carries all of the sugars in casein, it is known as glycomacropeptide (NAKANO & OZIMEK, 1998, 2000).

GMP is one of the various names given to the peptide formed by k-casein rupture. This peptide is also known as caseinomacropeptide (CMP) or casein-derived peptide (CDP). Usually GMP refers to the glycosylated form, due to its high carbohydrate content, and CMP to the peptide´s non-glycosylated form. Its composition varies and depends particularly on the whey source and on the technology used

for its isolation (MARTÍN-DIANA et al., 2006).

The glycosylated form represents 50 to 60% of total CMP and is composed of galactose, (Gal) N-acetylgalactosamine (GalNAc) and acetylneuraminic acid (NeuAc) (THOMÄ, KRAUSE & KULOZIK, 2006). The most predominant is NeuAc, known as sialic acid. GMP purified to 90% is highly glycosylated with 7 to 8% sialic acid (MARTÍN-DIANA, PELAEZ & REQUENA, 2003).

GMP is present in milk whey in concentrations of 1.2-1.5 g/L, it is rich in branched-chain amino acids (valine and isoleucine) and it lacks aromatic amino acids, including phenylalanine, tryptophan and tyrosine (OLIVA, ESCOBAR & PONCE, 2002). The same authors have informed that because of the absence of aromatic amino acids, GMP has no absorption at 280 nm. It is known that GMP is only detected in the range of 205-217 nm and differences in the absorption at 210/280 nm are frequently used to characterize GMP.

Several works have informed that the theoretical molecular weight of GMP is between 7 and 8 kDa. Some authors suggest that GMP has the ability of associating and dissociating under selected pH conditions. KAWASAKI et al. (1993a) proposed that the monomer of k-casein GMP of molecular weight 9 kDa is obtained at pH≤4 and the polymer of k-casein GMP of molecular weight between 45-50 kDa is obtained at pH higher than 4.

In 1998, NAKANO and OZIMEK suggested a study to establish GMP's molecular weight and whether it is in fact influenced by pH. Using chromatography in Sephacryl S-200 gel, the authors collected fractions that were monitored for sialic acid. The results suggested that GMP is an aggregate of three monomers and the molecular weight was not affected by changes in pH.

Currently there is an overall consensus that the experimental molecular weight is higher than the theoretical weight. MIKKELSEN et al. (2005) demonstrated in their studies that CMP's experimental molecular weight is higher than the theoretical one due to aggregation of CMP monomers of molecular weight up to 7 kDa. The authors suggest that CMP can aggregate up to 4 monomers which are resistant pH changes. The formation and conformation of these aggregates are influenced by CMP's properties in k-casein and by the fact that CMP bond to k-casein is reversibly influenced by pH.

MOLLÉ and LÉONIL (2005) reported that CMP has three potential phosphorilation sites and six potential glysosylation sites; it can present four genetic variants (A, B, C and E). The A and B forms are more common in bovine milk and are represented CMP_A and CMP_B. These forms differ in two amino acids: at position 136 the variant A has threonine and B, isoleucine; at position 148 variant A has an aspartic acid residue while variant B has an alanine residue (OLIVA, ESCOBAR & PONCE, 2002).

3 BIOLOGICAL ACTIVITIES AND NUTRICIONAL PROPERTIES

Currently various biological and physiological activities are being attributed to GMP, thus much attention is being given to its isolation and purification. Many studies in the last ten years have tried to establish the potential of GMP and its non-glycosylated form, CMP, in regulation of intestinal function (KORHONEN & PIHLANTO, 2006).

LIESKE, KONRAD and KLEINSCHMIDT (2004), studying the influence of pH and heating on CMP isolation by whey ultrafiltration, verified that CMP increases in heat sensibility at acidic pH, while heating at neutral pH produced neglectable changes in the peptide-sialic acid bond, thus a high degree of biological activity is obtained.

In a review on GMP's biological activities, BRODY (2000) highlights protection against toxins, bacteria, virus and immune system regulation as the most promising areas in the study of this peptide.

OLIVA, ESCOBAR and PONCE (2002) reported many biological activities attributed to GMP, among which are included: effect on gastrointestinal mobility, inhibition of the binding of the cholera toxin to it´s receptor, inhibition of the influenza virus hemagglutination, effect on the growth of lactic acid bacteria, effect on digestion, promotion of growth of bifidobacteria and antithrombotic activity.

The glycosidic structures of GMP are important for its biological activity (AIMUTES, 2004). KAWASAKI et al. (1992) suggested that the inhibitory effect of GMP upon the cholera toxin seems to be attributed to the terminal sialic acid.

GMP has also shown protection against influenza virus infection cells. KAWASAKI et al. (1993b) demonstrated inhibition of four species of human influenza virus, the effect was noticed in low GMP concentrations (80 mg/L).

There have been great research efforts on milk bioactive peptides, focusing cariogenic inhibition, inhibition of dental enamel demineralization e subsequent remineralization. AIMUTIS (2004) observed that GMP inhibited the growth of the cariogenic bacteria *Streptococcus mutans* and of other species. The author reinforces that the glycosidic structures bond to GMP are important for the biological activity including anticariogenic activity. GMP showed to inhibit dental enamel demineralization and also promoted remineralization. The same author supports the use of bioactive peptides in the production of commercial products such as tooth paste, gels and dental rinses. Among some developed products are: a formulation for dental remineralization, with the mixture of GMP and xylitol protected by the patent U.S. 6.207.138; a product for oral use containing the antibactericide GMP; and an anionic surfactant. There is also a protein hydrolysate described in patents U.S. 5.741.773 and U.S. 5.853.704 in which the formula of an anti-caries tooth paste is composed of a source of fluoride ions and GMP, that combined can enhance dental remineralization (ZHANG & GAFFAR, 2001, 1998a and 1998b).

There is evidence that GMP inhibits gastric secretion and stomach contractions. GMP also decreased the appetite of dogs (STAN et al., 1983). Some authors have suggested that GMP stimulates cholecystokinine release. This hormone is involved in the control of food ingestion and digestion in the duodenum of animals and humans (KORHONEN & PIHLANTO, 2006). The influence of GMP on weight gain was analyzed in rats treated with whey protein isolate (control), whey protein isolate+10% GMP, whey protein isolate+20%GMP and casein. The results showed a significant reduction in weight gain of rats treated with GMP, compared to the control. Furthermore, there was a 9% reduction in visceral fat of rats treated with whey protein isolate+20%GMP, compared to the control group (MCINTOSH, ROYLE & CLIFTON, 2005). Based on these studies commercial products containing GMP may be developed for appetite control and weight maintenance. On the other hand, GUSTAFSON et al. (2001), in a study with humans, evaluated the effect of CMP on satiety and satisfaction, measured by the amount of food ingested during the meal and by the subjective motivation (assessed using a questionnaire). There were prepared drinks containing 0.4% and 2.0% CMP, a drink containing only the vehicle (aspartame, citric acid, malic acid, water, fruit flavoring, red colorant and clouding agent to emulate protein) and water containing colorant and clouding agent. The researchers found that under experimental conditions CMP had no effect on the weight of food consumed at lunch or during the day. CMP also had no effect on the subjective satiety indicator and CMP administration before lunch had no effect in regulating food consumed during a short period of time. However, ANDERSON and MOORE (2004) suggest that before concluding that CMP does not contribute to satiety in humans, studies with higher doses of CMP must be evaluated, since the former study used drinks containing only 0.4 or 2.0 g of CMP.

Added to the biological activities mentioned above, GMP has special nutritional properties. It is rich in branched-chain amino acids and poor in methionine, thus it can be used as an ingredient in diets for hepatic patients (THOMÄ-WORRINGER, SORENSEN & LÓPEZ-FANDIÑO, 2006).

Because it lacks aromatic amino acids (phenylalanine, tyrosine and tryptophan) CMP is suggested as an ingredient in the diet of phenylketonuric patients (OLIVA, ESCOBAR & PONCE, 2002).

GMP is also recognized for enhancing the absorption of minerals such as calcium, iron and zinc. KELLEHER et al. (2003) verified in their studies that GMP supplementation enhanced zinc absorption, allowing a reduction of this mineral in infant formulas.

4 FUNCTIONAL TECHNOLOGICAL PROPERTIES

The development of innovative foods with an additional health benefit is a goal for the food industry. The characteristics of CMP not only permit medicinal and dietary applications, but also give the molecule a great potential as a structural agent for food, since its glycosidic structure suggests emulsifying and foaming properties (KULOZIK & GUILMINEAU, 2003).

GMP is an acid peptide with pI between 4 and 5, highly soluble and heat stable (THOMÄ-WORRINGER, SORENSEN & LÓPEZ-FANDIÑO, 2006).

GMP showed to be stable in the pH range of 1 to 10, with minimal solubility (88%) between pH 1-5 and maximum (98%) between pH 5-10. The emulsifying activity was stronger at acid pH rather than alkaline. After letting the emulsion stand for 24 hours and heating there was observed a decrease in the emulsifying activity (22-60%) at pH below 4 (CHOBERT et al., 1989).

MARTÍN-DIANNA et al. (2006) studied the viscoelastic properties of CMP isolated from milk whey at different values of pH (2, 4, 7 and 10), ionic strength (0; 0.2; 0.4 and 0.7M in NaCl) and protein concentration (50, 100 and 200 gkg^{-1}), at temperatures from 10 to 90°C. The authors found that the best condition to maintain constant relative viscosity levels with a raise in temperature above 75°C is at pH 7. In high protein concentrations (200 gkg^{-1}) rheological changes caused by heat occur at moderate temperatures (40-50°C) with no considerable difference in viscosity.

WONG, NAKAMURA and KITTS (2006) determined the functionality, foaming capacity and emulsifying activity of GMP after conjugation to fatty acids. The authors observed that the foaming capacity was lost, whereas the emulsifying activity enhanced.

The addition of CMP to fermented goat milk favored gel formation in a more orderly and structured manner compared to the addition of whey protein concentrate (MARTÍN-DIANA, PELAEZ & REQUENA, 2003). However, VEITH and REYNOLDS (2004) verified in their work that the presence of CMP had a negative impact on gel strength and water retention capacity. On the other hand, MARTÍN-DIANA, FRIAS and FONTECHA (2005) studied CMP of cow, ewe and goat cheese whey, concluding that CMP had an emulsifying activity more stable to pH variation, compared to whey protein concentrate. This suggests the possibility to use CMP as an emulsifier in foods that undergo great pH variation during processing, such as fermented dairy products.

CMP obtained from goat milk was modified with lactose through Maillard reaction under relative humidity 44% and temperature of 40°C for periods of 0 to 11 days, thus obtaining different forms of lactosylated CMP. At these conditions, the most abundant form of lactosylated CMP was the monolactosylated (55-60%), followed by the di-, tri- and tetralactosylated species. Solubility, heat stability and emulsifying capacity of native and modified CMP were investigated. Lactosylation enhanced emulsifying capacity but did not improve the outstanding solubility and heat stability of native CMP (MORENO, LÓPEZ-FANDIÑO & OLANO, 2002).

5 ISOLATION AND PURIFICATION METHODS

Lately there has been an effort to develop large scale GMP production processes that are able to maintain the molecule's biological and nutritional properties. Ion-exchange resins and ultrafiltration are among the methods used to obtain GMP from milk whey or whey protein concentrate (CASAL et al., 2005).

The various methods for GMP isolation and purification presented in this paper consist of one process or of a combination of different processes and can be divided into three main categories: 1) selective precipitation induced by adjustment of the physical properties of the solution (heating, alcoholic precipitation, precipitation using trichloroacetic acid or using acetic acid); 2) membrane filtration based mainly on different membrane sizes and load (dialysis, ultrafiltration, microfiltration, reverse osmosis, electrodialysis) and 3) selective adsorption (ion-exchange chromatography).

In the patent U.S. 4.042.575, EUSTACHE (1977) proposed a method to extract glycoproteins and sialic acid from whey. Until then ultrafiltration hadn't been used to separate and obtain specific proteins. The patent reports the process in detail, that in short consists of whey ultrafiltration, thermal flocculation of the proteins in the retentate followed by phosphotungstic treatment of the supernatant, acid hydrolysis of the precipitate and treatment of the hydrolysate supernatant to extract the sialic acid. This process can yield 13 g of extremely pure sialic acid from 900 liters of milk whey.

After using dialysis to isolate GMP from sodium caseinate and skim milk hydrolysate, MORR and SEO (1988) purified the molecule using three chromatographic methods: exclusion chromatography (Sephadex G-10 and G-25 columns), ion-exchange chromatography (DEAE-Sephadex column) and affinity chromatography (Con A-Sepharose column). Analysis using high performance liquid chromatography (HPLC) showed a peak with molecular weight of 33 kDa while electrophoresis revealed a group of peptides of heterogeneous size with molecular weight of 18 kDa or lower.

The process described by TANIMOTO et al. in the patents E.P. 0393850A2 of 1990 and U.S. 5.075.424 of 1991, supports GMP production adjusting the peptide solution to pH<4, followed by ultrafiltration in a membrane with a cut off of 10-50 kDa and concentration of the filtrate in a membrane of cut off lower than 50 kDa to obtain a desalted concentrate. According to the inventors, GMP produced following this method may be used by the food industry. KAWASAKI et al. (1993a) used this method and obtained a purified product with phenylalanine content of 0.6% (w/w) and 81% purity.

The method patented by DOSAKO, NISHIYA and DEYA (1991) in U.S. 5.061.622 reveals an efficient process for GMP production on an industrial scale, with 80% minimum purity, 5% minimum sialic acid content and ash content above 10%. The raw material can be acid casein, sodium caseinate or calcium caseinate. The effluent free from whey protein and lactose is acidified and calcium phosphate is removed by centrifugation or filtration. The effluent is then desalted with an ion-exchange resin, electrodialysis or reverse osmosis, and finally concentrated by vacuum evaporation. When a reverse osmosis membrane is used, concentration and desalting may be performed together.

According to KAWASAKI and DOSAKO (1991), the method patented in E.P. 0488589A1 proposes a GMP production process in which the material is put in contact with an ion-exchanger. The fraction that is not adsorbed is collected, adjusted to pH below 4 and treated by ultrafiltration using a membrane with cut off of 10-50 kDa. The filtrate is then passed through a membrane with cut off below GMP's molecular weight. The resulting product has purity between 55 and 88%.

A novel method to isolate CMP from milk whey was proposed by SAITO, YAMAJI and ITOH (1991). The authors reconstituted powdered whey (10% v/v), adjusted the pH to 6 and heated the solution for one hour at 98°C. After cold precipitation with ethanol (50% v/v), the precipitate was separated by centrifugation and the supernatant was applied to a DEAE-Toyoperarl 650M column. The elution was performed with a solution of ammonium bicarbonate 0.3 mol/L. The yield using this process was of 1.1 g of CMP for 100 g of powdered whey.

In 1992, TANIMOTO et al. used whey of lactic casein and proposed a large scale GMP production method. The whey was filtered, desalted and lyophilyzed resulting in a GMP preparation with phenylalanine content of 2.4% (w/w). GMP was purified by ion-exchange chromatography, using Q-Sepharose®, presenting phenylalanine levels of 0.9% (w/w).

In the GMP production process reported in the patent U.S. 5.216.129 by BERROCAL and NEESER (1993), the proteins of a whey concentrate partially free from lactose are flocculated. The supernatant is collected and concentrated by ultrafiltration, producing a retentate. This fraction is treated with ethanol, which precipitates the residual soluble protein. The supernatant is then collected and dried. From 20 kg of whey protein concentrate dispersed in 180 liters of water, 290 g of GMP is obtained, with 10.9% sialic acid and 8.4% protein.

In patent WO 94/159242, NIELSEN and TROMHOLT (1994) reveals a method of producing GMP from whey by ultrafiltration in a membrane with a 16-20 kDa cut off, followed by heating the retentate for 15 minutes, adjusting the pH to 4-5, filtration and collection of GMP from the filtrate. With

this process the purity is of 70% and phenylalanine of 1/3 compared to the starting material (the exact concentration is not informed). The authors affirm that this method results in GMP that can be used in food for specific needs and in medications for diarrhea caused by viral infection.

Investigating the viability of producing GMP by ultrafiltration of sodium caseinate, CHU, MACLEOD and OZIMEK (1996) tested a combination of membranes: a) 50 and 10 kDa; b) 30 and 10 kDa and c) 10 kDa. The retentates obtained using 30 and 50 kDa membranes resulted in higher GMP recovery, with GMP representing 50.9% of the protein recovered by the 30 kDa membrane.

The GMP purification process for scale production proposed by AYERS et al. (1998) in the patent WO 98/14071 starts with milk derived products with phenylalanine concentration of 0.5% (w/w). The GMP obtained can be used as a supplement for phenylketonuric patients. The process consists in using an ion exchanger (QA GiboCel ™, Life Techonologies Ltd) under conditions that GMP is adsorbed, followed by elution of GMP, removing impurities by numerous processes and recovery of purified GMP. At pH 5.1 there was elution of 91% of the GMP.

The inventors of patent U.S. 6.462.181 B1, HOST and CHATTERTON (2002) perfected the process cited in patent E.P. 0393850A2, by TANIMOTO et al. (1990), adjusting the pH of the starting material (whey protein concentrate) to below 4, followed by cold ultrafiltration and concentration in a special membrane with high mechanical stability after 1 year of use 24 hours a day, 7 days a week. The inventors sustain that the process can be used on industrial scale without fouling problems. They inform that cold filtration preserves the membrane and also produces different retention, with lower permeability. This is proved in two experiments with and without heating, in which the GMP (measured as non-protein nitrogen) content is increased from 24.6 to 30.7% when no heat is applied.

Anion-exchange chromatography was used by NAKANO and OZIMEK (1999) to purify GMP from the non-dialyzable fraction of whey using a DEAE-Sephacel column at two pH values (6.4 and 3.0). The column at pH 3.0 resulted in GMP of high purity and yielded 1 g per liter of whey. The authors conclude that chromatography with DEAE-Sephacel at pH 3.0 is an easy method that can be applied in large scale for GMP separation from whey proteins.

Separation of immunoglobulin G and GMP from milk whey was investigated by XU et al. (2000), who used anion-exchange chromatography with resin IRA93 and Amicon YM100 membrane. Since GMP is negatively charged at pH 4.7, the IRA93 resin adsorbed GMP.

ETZEL (1999) presents a process to produce GMP with nutraceutical properties from whey using two anion exchangers of opposite polarity in series. The process is reported in the patent U.S. 5.968.586 and according to the author the product obtained contains 4% of the total amount of the aromatic amino acids phenylalanine, tryptophan and tyrosine residues.

DOULTANI, TURHAN and ETZEL (2003) also demonstrated the viability of using two anion exchangers of opposite polarity in series to recover simultaneously whey protein isolate and GMP. To recover the whey protein isolate a column was packed with a cation exchanger (SP Sepharose Big Beads, Amersham Biosciences) and to recover GMP a column was packed with an anion exchanger (Q Sepharose Big Beads, Amersham Biosciences). The columns were eluted with non-expensive food grade buffer. The recovery was of 92% of whey protein and 96% GMP.

In an attempt to purify GMP from a solution of caseinate hydrolyzed by chymosin, NAKANO and OZIMEK (2000) used processes involving a) exclusion chromatography with Sephacryl S-200 at pH 7 to obtain the GMP fraction; b) addition of acid solution, pH 3.5 to precipitate contaminating proteins and peptides and c) chromatography in Sephacryl S-200 at pH 3.5. The preparation was considered to have high purity with only traces of arginine, histidine, phenylalanine and tyrosine. In the same year, the authors purified GMP from non-dialyzed fractions of sweet whey using hydrophobic interaction chromatography in a phenyl-agarose column. There was obtained approximately 1.6 g of GMP for one liter of sweet whey.

A process to produce substantially pure GMP with nutraceutical properties is presented by ETZEL (2001) in patent U.S. 6.168.823 B1. It is based on affinity chromatography, with contact of whey with an anion exchanger. The novelty of this invention is the discovery that proteins are immobilized

by metallic anions, but not CMP. The patent describes in detail the production of CMP that can be used for medical or food purposes. The pH of the whey is adjusted to 4-6 and passed through a 0.7 μm filter. The author informs that the advantage of the process is the use of high temperature (35 to 60ºC) increasing separation efficiency. After contact with an anion exchanger two fractions are produced: whey protein enriched with CMP and a whey solution without CMP. The enriched fraction is put in contact with a metal chelating adsorbant, the contaminating peptides and proteins are adsorbed, purifying the CMP fraction.

NAKANO et al. (2002) purified GMP from sweet whey achieving undetectable phenylalanine levels. Sweet whey was deproteinyzed with trifluoracetic acid followed by chromatography in Sephacryl S-200. The GMP was considered to be of high purity, with molecular weight of 31 kDa and with undetectable levels of phenylalanine, tyrosine and arginine.

VASBINDER, ROLLEMA and KRUIF (2003) isolated GMP using precipitation with acetic acid/ sodium acetate and precipitation with trichloroacetic acid (TCA) at 2%, 8% and 12%. The HPLC chromatograms of the supernatants using acetic acid/sodium acetate and using TCA at 2% were identical in form and position. However increasing TCA concentration caused a decrease in the peak areas. Non-glycolysated GMP variant B disappears completely after precipitation with 8% and 12% TCA. Part of the non-glycolysated GMP variant A precipitates using 8% TCA and practically disappears with 12% TCA. Increasing TCA concentration the total peak area decreases 60 to 80% using 8% and 12% TCA, respectively, compared to 2% TCA.

LI and MINE (2004) compared the chromatographic profile of GMP isolated by three methods: fractioning with TCA, precipitation with ethanol and ultrafiltration of whey protein isolate. The authors verified that pre-treatment with TCA recovered only glycosylated GMP and eliminated all other proteins, but the recovery was low, only 6.7% of the initial GMP. Ethanol precipitation recovered 20.4% of GMP in the initial whey isolate, of which 75.7% was in the glysolylated form. Utrafiltration was the most effective method to recover GMP, with 33.9% recovery, of which 81.6% was glycosylated.

In an attempt to use an abundant residue of the fishing industry, NAKANO, IKAWA and OZIMEK (2004) developed a method to isolate GMP using chitosan as anion exchanger. Shrimp husks were used to prepare two chitosan resins, one with primary amine as main functional group (resin A) and the other with secondary amine (resin B). These resins were tested as adsorbants to isolate GMP from whey and compared to a commercial anion exchanger. The most important finding of this study was that the binding of GMP was much higher in resin A than in B. The authors propose tests for industrial large scale production of GMP. The amino acid analysis suggests that the isolated GMP can be using replacing milk whey in various food products including infant formulas, bakery products and drinks.

Researching a novel method to separate CMP from whey proteins, TOLKACH and KULOZIK (2005) included a pre-treatment of whey protein concentrate with the enzyme tranglutaminase, followed by microfiltration. CMP's amino acid sequence includes two residues of glutamine and three of lysine so the peptide is binded to transglutaminase. Whey proteins are less sensible to the enzyme because of their globular conformation. CMP with a covalent bind can be removed by microfiltration. It was demonstrated that CMP is a good substrate for transglutaminase and it binds selectively between the glutamine and lysine residues.

According to TOLKACH and KULOZIK (2005), there are currently two main methods to separate GMP from whey proteins using ultrafiltration. The first one is described by KAWASAKI et al. (1993a) and reveals the heterogeneity of GMP regarding its molecular mass with pH variation. Membranes with a higher or lower cut off can be used, making it possible to increase operational flow. The second method is described by MARTÍN-DIANA and FONTECHA (2002) and is based on the heat stability of CMP compared to other whey proteins. This process includes acidification, heating and ultrafiltration of milk whey to obtain powdered CMP with a protein content of 75-79%. The authors recovered CMP from the whey protein concentrate of cow, ewe and goat milk, in levels between 71 and 76% and purity of 75 to 90%. Using this technique the heat treatment at 90ºC for 1 hour causes complete denaturation and

aggregation of whey proteins. These can be removed by centrifugation at 5200 g, 4ºC for 15 minutes. The supernatant containing CMP can be concentrated by ultrafiltration with a 10kDa membrane. The disadvantage of this method is that part of the whey proteins loose their functionality due to denaturation.

6 CONCLUSION

The possibility of having new dietetic and pharmaceutical products is promising and GMP has been in evidence with applications in dietetic supplementation, functional foods and pharmaceutical preparations. However, whether GMP is beneficial and desirable as a food component still must be examined. Possible adverse effects associated to GMP must also be considered if it is to be used in concentrations higher than those that are normally ingested in dairy products.

Although GMP is a promising compound that has diverse biological applications, most data regarding its bioactivity are results of *in vitro* or animal studies. In order to commercialize products containing GMP with a health benefit claim, *in vivo* studies with humans must be conducted. This can also be extended to the relation between structure and biological activity, linking the glycolsylated form to biological functions.

Numerous processes were developed in the last years attempting to purify GMP. Some allow large scale production for its use as a food ingredient. Despite this progress, the technological aspects are limited to GMP isolation and purification, with little information regarding its application in food, interaction with other components and chemical stability under different processing conditions.

RESUMO

REVISÃO: ISOLAMENTO E PURIFICAÇÃO DO GLICOMACROPEPTÍDEO DO SORO DE LEITE

Este trabalho teve como objetivo efetuar revisão dos métodos de isolamento e purificação do glicomacropeptídeo (GMP), peptídeo formado durante o processo de coagulação enzimática do leite pela quimosina. Também foram abordados aspectos como estrutura, composição, atividades biológicas e propriedades nutricionais e tecnológicas do GMP. Esse peptídeo, conhecido por ser um composto bioativo, apresenta potencial elevado como agente estrutural do alimento. Conclui-se que os vários métodos relatados para o isolamento e purificação do GMP utilizam de forma isolada, ou combinada, processos que podem ser classificados em três categorias: precipitação seletiva induzida por ajuste das propriedades físicas da solução; filtração por membrana baseada principalmente em diferenças de tamanho e carga; e adsorção seletiva. Em relação às atividades biológicas pode-se entender o GMP como composto promissor, mas pesquisa adicional é necessária para definir as quantidades para se atingir a eficácia e permitir alegações de funcionalidade. Quanto às aplicações tecnológicas desse peptídeo são poucas as informações quanto à sua adição em alimentos, interações com outros componentes e estabilidade química sob diferentes condições de processamento.

PALAVRAS-CHAVE: SORO DE LEITE; GLICOMACROPEPTÍDEO; ATIVIDADES BIOLÓGICA.

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