DEVELOPMENT OF FUNCTIONAL FOOD INGREDIENT AS A SOLUTION FOR PROTEIN-ENERGY MALNUTRITION; AN AMINO ACID SOURCE WITH LOW-PHENYLALANINE CONTENT

A Thesis Submitted to
the Graduate School of Engineering and Science of
İzmir Instıtute of Technology
in Partial Fulfillment of the Requirement for the Degree of

MASTER OF SCIENCE

in Food Engineering

by
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July, 2015
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ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my supervisor Professor Şebnem Harsa for her supervision, support, patience, encouragement, favor and in confidence in me during MSc research. I also want to thank her for giving me this opportunity to make real my ideas. I also would like to thank Professor Figen Korel, Professor Muhsin Çiftçioğlu and Professor Yekta Göksungur for their valuable supports.

I must also thank to Specialists Yekta Günay Oğuz and Dane Rusçuklu from Biotechnology and Bioengineering Aplication and Research Laboratory, for their support, patience and favor.

I am equally grateful to Specialist Burcu Okuklu Öztürk, and Research Assistants Ece Sürek, Gökçen Kahraman and Merve Şamlı. They always helped me when I needed, in personal and professional matters.

Burçin, Elif, Kübra, Görkem and Zeynep Nale could not be forgotten. To them, who always makes me laugh even if they are miles away, I feel like they are always there for me whenever I need.

I also thank Specialist İlknur Şen for her helps during RP-HPLC analysis and Yasemin Parlar for her helps during cheese making.

A special thanks to Associate Professor Elçin Bora who encouraged me for an academic career at Izmir Institute of Technology.

I would like to thank Ömer Uysal from Intermak A.Ş. and İbrahim Dalar from Maysa Gıda for providing rennet to use in whey production.

I also thank to Bilgin Oğuz and Fatma Bayraktar from Türer A.Ş. for kindly providing to caprine whey.

I must also thank to my spiritual support team, my aunts Saniye, Suzan, Sevim, Ayşe, Leyla, Emine, Birnur and Mukadder for always remanding me about everything will be fine.

Finally, an endless thanks to my father Ali Akın, my mother Hayriye Akın and grandmother Sabriye Tanınmış for support me to do what is right for me. They always helped to find my way during hopeless moments and always love in me. I feel safe whenever with them. So glad I have them.

Lastly, I thank everyone who helped and supported me during this thesis and whole my education life.

ABSTRACT

DEVELOPMENT OF FUNCTIONAL FOOD INGREDIENT AS A SOLUTION FOR PROTEIN-ENERGY MALNUTRITION; AN AMINO ACID SOURCE WITH LOW-PHENYLALANINE CONTENT

Casein glycomacropeptide (CGMP) is one of the whey protein which constitutes 12-20% of whey proteins approximately. It has various functional and biological activities. Such as growth-promoting effects on bifidobacteria, suppression of gastric secretion and the inhibition of viral or bacterial adhesion to intestinal epithel cells, inactivation toxins of *Escherichia coli* and *Vibrio cholerae*, inhibition adhesion of cariogenic bacteria with use in toothpastes. It also has good foaming emulsifying agent properties. The objective of this thesis was to develop a new functional food ingredient suitable for use by individuals with Phenylketonuria (PKU) that causes protein malnutrition. In line with this objective, CGMP was used as a protein source. It was isolated from caprine whey by ultrafiltration process. This process was applied with different parameters to obtain CGMP with high purity and yield. Protein fractions and Phenylalanine and tyrosine contents of CGMP isolates were determined by RP-HPLC. Some compositional analysis was carried.

ÖZET

PROTEİN-ENERJİ MALNÜTRİSYONUNA ÇÖZÜM GETİREBİLECEK FONKSİYONEL GIDA BİLEŞENİ GELİŞTİRME; DÜŞÜK FENİLALANİN İÇEREN AMİNO ASİT KAYNAĞI

Beslenme yetersizliği (malnütrisyon) bulunan bireylerin protein gereksinimlerini kısmen karşılayacak yeni bir fonksiyonel gıda bileşeni geliştirilmesi amaçlanmaktadır. Bu çerçevede, protein kaynağı olarak Kazein glikomakropeptit' in (KGMP), peynir üretimi yan çıktısı olan ve kirletici atık niteliği taşıyan peyniraltı suyundan (PAS) izole edilerek kullanılması planlanmaktadır. Kazein glikomakropeptit (KGMP), alfa-laktalbumin ve beta-laktoglobulin den sonra PAS' nda en yüksek miktarda (/oranda) bulunan proteindir. Yapılan araştırmalar ile KMP'in, gastrik salgıyı engelleme, bifidobakteriyal gelişmeyi destekleme, *Vibrio cholera* ve *Escherichia coli* enterotoksinlerini bağlama, viral ve bakteriyal adhezyonları inhibe etme, alerjik reaksiyonlara karşı bağışıklık sistemini destekleme, antihipertansif etki ile kan dolaşımını düzenleme, diş yüzeyinde plak oluşumunu, diş çürümesini ve diş yüzeyine kariyojenik bakterilerin tutunmasını önleme, lipid birikimini azaltarak ve antioksidan kapasitesini yükselterek anti-obezite etkisi gösterme gibi besinsel ve tıbbi özelliklere sahip olduğu ve bir biyoaktif peptit gibi davrandığı belirlenmiştir. Bunun yanı sıra Kazeinomakropeptit yapısında doğal olarak fenilalanin amino asidini içermediği bilinen tek protein kaynağıdır. Çalışmada, keçi peynir altı suyundan izole edilecek KGMP kullanılarak malnütrisyonlu ve fenilketonurili bireylerin kullanımına uygun yeni bir gıda bileşeni geliştirilecektir. Böylece yan ürün veya atık niteliği taşıyan PAS'nun ekonomik bir değere dönüştürülmesi amaçlanmaktadır.

This work is dedicated to
Cavit, Süleyman, Ahmet, Şükrü and Mehmet
Rest in peace.....

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LIST OF SYMBOLS AND ABBREVIATIONS

α-lac	Alpha-lactalbumin
β-lg	Beta-lacctoglobulin
RP-HPLC	Reversed Phase High Performance Liquid Chromatography
Phe	Phenylalanine
PKU	Phenylketonuria
Tyr	Tyrosine
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
CGMP	Casein glycomacropeptide
Eq	Equation
nm	Nanometer
h	Hour(s)

CHAPTER 1

INTRODUCTION

Malnutrition can be defined as " a state resulting from lack of uptake or intake of nutrition leading to altered body composition (decreased fat free mass) and body cell mass leading to diminished and mental function and impaired clinical outcome from disease" (Sobotka, 2012). Sources of adult malnutrition are shown in Figure 1.1.

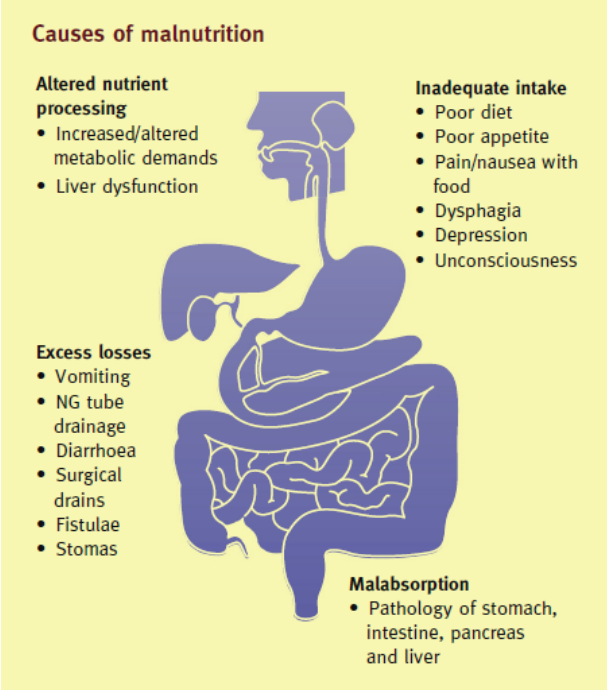


Figure 1.1 Causes of adult malnutrition
(Source: Saunders et.al., 2014)

Inadequate nutrition intake is probably the most important factor, what causes malnutrition (Jesson and Leroy, 2015; Saunder et.al., 2014; Walton and Allen, 2011).

Malnutrition is a global problem that affects millions of children and adults. In the developing countries, 20% children are underweight and 19 million (3.5%) have severely malnutrition (Walton, and Allen, 2011). It is correlated with more than 1/3 of all deaths in less than 5 years age children between 1990 and 2015, worldwide (Jesson and Leroy, 2015). On the other hand, malnutrition has a serious financial burden to publics. British Association of Parenteral and Enteral Nutrition (BAPEN) has declared that the

expenditure related to diseases induced malnutrition was more than £13 billion in 2007 in the UK and this value is greater than costs related with obesity (Russell and Elia, 2010).

3 million children were also living with HIV in Saharan Africa and, in total of 3.2 million children were also living with HIV worldwide in 2013. In addition, 240.000 children recently infected every year. Jesson and Leroy (2015) reported that HIV infection increases the prevalence of malnutrition. Malnutrition has similar impact as HIV on the immune system, and increases the progression of HIV disease. WHO (2009) indicates that, a child who infected with HIV needs 10% more energy than a child who are not infected with HIV. If child is malnourished and infected with HIV, this energy needs are increased by 50 to 100% (Irlam et.al., 2013; WHO, 2009).

As a result of research conducted in rats, it was reported that prenatal protein malnutrition (PPM) leads to irreversible alterations in behavior and brain (Amaral et.al., 2015). Rats with prenatal protein malnutrition have been shown anatomical, neurophysiological, behavioral, and morphological defects, even after postnatal nutritional treatment (Morgane et.al., 2003; Galler et.al., 1996; Tonkiss and Galler, 1990). These neuroanatomical and neurophysiological alterations are caused to augment neuronal inhibition in the hippocampus (Lister et.al. 2011; Chang et.al., 2003). PPM also caused to changes in neurotransmitter release (Mokler et.al.; 2007) and in activation of genes that are responsible for stress in the prefrontal cortex (PFC) (Rosene et.al.; 2004). These results can be accepted as an evidence that PPM in humans is correlated to neuropsychiatric and behavioral diseases and to cognitive impairment late in life, with antisocial personality diseases, schizophrenia and attention defects (Susser and St Clair, 2013; Galler et.al., 2013, 2012; St Clair et.al., 2005; Susser et.al., 1996). Although neurological and behavioral effects of malnutrition have been well known, there is no sufficient information about the molecular mechanism of these effects (Amaral et.al., 2015).

It was reported that older people who care in hospitals or homes 28-42% were malnourished (Russell and Elia, 2012, 2010, 2008). Sensory losses, anorexia, swallowing and chewing problems, and chronic or acute disease may affect dietary intake and cause to nutritional deficiency and malnutrition in older people. Malnutrition increases the risk for mortality in older people (Rasheed and Woods, 2013; Stratton et.al., 2003). However this risk can be decreased with nutritional rehabilitation. Stratton and Elia (2007) reported that determination of daily nutrient intake and regulation of this value can reduce the malnutrition by 70% and mortality decreased by 40% in malnourished patients.

Digestion and absorption problems (Celiac, Malabsorption, Phenylketonuria etc.) lead to malnutrition. Phenylketonuria (PKU) is one of the protein metabolism disorder. Individuals with PKU cannot breakdown Phenylalanine amino acid (Phe) in their metabolism, hence they should keep a restricted diet. This study is intended to develop a functional foods which are suitable for diet of individuals with Phenylketonuria.

1.1. Phenylketonuria

Phenylketonuria (PKU) is a genetic recessive disease, affecting roughly one in 15.000 births (Scriver and Kaufman, 2001). PKU is caused by a mutation in phenylalanine hydroxylase (PAH) gene (Figure 1.2). This mutant gene alters the structure of Phenylalanine (Phe) hydroxylase enzyme, hence unable to break down Phe to Tyrosine (Tyr) sufficiently. Phe begins to accumulate in blood, and brain and leads to toxic level (Scriver and Kaufman, 2001; Smith and Lee, 2000). This situation cause mental retardation, convulsions, neurological damage, behavior problems, skin rash and musty body odor.

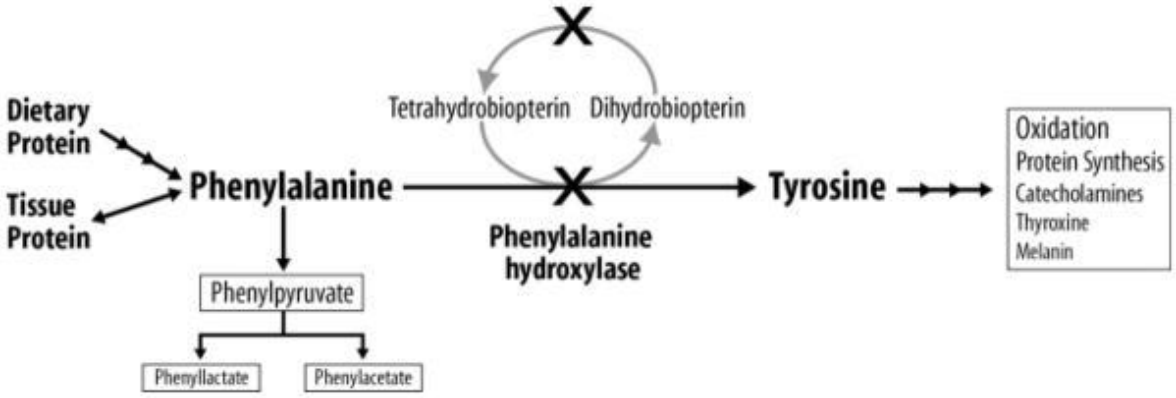


Figure 1.2. Mechanism of mutated PAH gen
(Source: van Calcar and Ney, 2012)

Phenylalanine is an indispensable, aromatic amino acid (Figure.1.3) which is essential for protein synthesis (Donlon et.al., 2008). *In vivo* studies reported that 27–41% of L-phenylalanine is transformed into tyrosine within 5–8 h of intake (Basile-Filho et.al., 1998; Sanchez et.al., 1996).

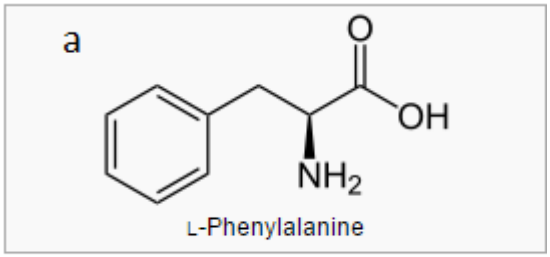


Figure 1.3. Structure of Phenylalanine.
(Source: https://en.wikipedia.org/wiki/Phenylalanine)

The treatment for PKU is consisted to a lifelong diet therapy (Scriver et.al., 2001; Bickel et.al., 1953). Classification of foods determined according to PKU diet is presented in Table 1.1. However this restricted diet inhibits the intake of undamaged protein sources especially after the age of 2 years and obliges individuals to consume specific medical foods consisted of Phe-free amino acids, minerals and other nutrients for a proper functionality of metabolism (WHO, 2007; Arnold et.al., 2002). Otherwise nutritional insufficiencies caused to decline in linear growth in some children with PKU.

Table1.1 Classification of foods for PKU diet*

Permitted Foods	Limited Foods	Banned Foods
Corn starch	Vegetables	Dairy products
Plain Turkish Delight	Fruits	Egg
Plain hard candy	Bakery products	Meat and meat products
Black/Linden/Sage Tea	Olive	Bread
Oil	Butter	Dried Nuts and Fruits
Sucrose	Margarine	Legume
Apple Juice	Honey	Processed foods
Compote	Pekmez	All foods, drinks, candies and gums that contains Phe and Aspartame
Pop		
Cola drinks		

*Rutherford and Poustie, 2005; www.pkuaile.com

Dhont et al. (1995) was reported that, relaxation of diet normalized the height of French individuals with PKU. In a study, German children lost their height significantly in the first year of life and got longer to expected normal height in the second year (Weglage et.al., 1994). In another study, height acceleration declines in the second year of life and could not catch the normal growth in later years (Schaefer et.al., 1994). Protein deficiency causes decline in linear growth of PKU patients. Since the protein sources generally contain Tyr and Phe, the source of protein is the main cause of the PKU.

However, poor growth in 2 children with phenylketonuria who got a lower dietary protein intake but in other children with phenylketonuria taking a higher protein intake.

Researches about protein requirements are incomplete and sufficient yet (WHO, 2007). Therefore there is no precise information about the protein needs of PKU. In addition, it is also difficult to determine Phe requirements of individuals with PKU. Courtney et al. (2002) showed that Phe requirements of 6-13 years age children with PKU are 14 mg/kg/day. On the other hand, a daily allowable consumption of Phe for adults (age > 19) is varied between 290 and 1200 mg/d for male or 220 and 770 mg/d for female (Acosta and Yanicelli, 2001).

It is very difficult to implement a restricted diet throughout life, but disobedience can be caused by neuropsychological disabilities. Increasing the variety of palatable foods, that contain Phe-free protein, make easier to follow the diet. Casein glycomacropeptide (CGMP) is the only protein that does not contains any Phenylalanine naturally. GMP has been used as a safety alternative among any amino acid diet and can improve the quality and acceptability of foods products. In addition, when synthetic amino acid based diet supplies almost 80% of protein requirements, a CGMP based diet supplies almost 70% of protein requirements from GMP, fruits and vegetables and almost 30% of protein requirements supplied from synthetic amino acids. There is a few study about development of foods that contains CGMP as a protein source. Lim used a patented commercial CGMP BioPURE-GMP^{TM} (Davisco Foods International, Inc., Minnesota, USA) to formulate low–Phe foods. Puddings contain strawberry and fruit leather, chocolate beverage, orange sports beverage snack crackers are the foods formulated with CGMP in that study (Lim et.al., 2007). As a result of sensory analysis, it was determined that all developed foods were acceptable. A variety of acceptable, new products can be produced from CGMP that fulfill the nutritional requirements. Whey is an only and rich source of CGMP. CGMP depends on the whey composition, production methods and food quality.

1.2. Whey

Whey is the most valuable and widely accepted by-products of the dairy industry. It is the soluble fraction of milk and is released by the action of rennet to casein during the production of casein or cheese. Sweet whey (cheese whey) is rennet-driven coagulated whey and lactic acid or mineral coagulated casein is named as acid whey. Whey was defined as pollutant (Kaur et.al., 2009). Since its biological oxygen demand is highly substantially (chemical oxygen demand = 60 g/kg; Siso,1996), and because of this reason, annihilation process can be expensive. Lactose content of whey is high and it is the main cause of the pollution.

Caprine dairy products are substantial in human nutrition. Most malnourished and starving people are feeding with caprine products instead of bovine products in developing countries (Haenlein, 2004). Caprine milk is considered as less allergic than bovine milk. Individuals suffering from gastrointestinal diseases and cow milk allergies prefer consuming caprine milk products. There have been an increasing interest trough caprine dairy products that is growing a new market share in developed countries. Caprine whey, having unique protein composition, is composed of β-lactoglobulin, α-lactalbumin, serum albumin, lactoferrin, casein glycomacropeptide, immunoglobulins and lactoperoxidase (Table 1.2) (Hernández-Ledesma et.al., 2011). This composition is affected from the time of the year, the quality of processing, the type of feed, the stage of lactation and the type of whey (sweet or acid). The whey proteins from caprine have a superior nutritional value than proteins from cows (Moulin and Galzy, 1985).

Table1.2. Whey proteins and their basic properties*

Protein	Concentration(g/l)	Molecular mass (kDa)	Isoelectric Point
β-lactoglobulin	2.7	18.277	5.2
α-lactalbumin	1.2	14.175	4.5 - 4.8
Glycomacropeptide	1.2	6.7	3.2
Immunoglobulins	0.65	25.0-7000.0	5.5 – 8.3
Bovine serum albumin	0.4	66.267	4.7 – 4.9
Lactoferrin	0.1	78.0	9.0
Lactoperoxidase	0.02	89.0	9.5

*Tarhan, 2013; Madureira et.al., 2007

Whey can be used as a raw material in food industry. Whey protein isolates (WPI) and whey protein concentrates (WPC) are used for specific diets in which high protein content is needed, such as baby foods and sports drink. Whey proteins have various biologic features such as antioxidant activities, antimicrobial properties, anti-cariogenic and immunomodulatory activities (Table 1.3). Whey proteins also presence functional features such as emulsifying, gelation and foaming (Neelima et.al., 2013). The investigations on the functional and biological features of whey proteins isolated from bovine whey/milk are well documented in literature. Nevertheless only a few studies were focused on the functional and biological properties of whey proteins from caprine. There is a need for and extensive research to understand the functionality of caprine whey and implications for potential food applications.

Table 1.3. Biological features of whey proteins (CSID: chronic stress-induced disease)

Protein	**Biological Features**	**References**
β-lactoglobulin	✓ Antihypertensive effect ✓ Hypocholesterolemic effect ✓ Antimicrobial activity ✓ Anti-HIV activity	Nagaoka et.al. (2001) Pellegrini et.al. (2001), Berkhout et.al. (2002), Ouwehand and Salminen (1998)
α-lactalbumin	✓ Treatment of CSID ✓ Lactose synthesis ✓ Anti-cariogenic activity	Ganjam et.al. (1997) Markus et.al. (2002) de Wit (1998)
Immunoglobulins	✓ Antibacterial activity ✓ Activity of opioid ✓ Antifungal activity	Freedman et.al. (1998) Sharpe et.al. (1994) Okhuysen et.al. (1998)
Bovine serum albumin	✓ Binding of fatty acid ✓ Anti-cariogenic activity ✓ Anti-mutagenic activity	Walzem et.al. (2002) Laursen et.al. (1990) Bosselaers et.al. (1994)

As seen in Table 1.2, β-lactoglobulin (β-lg) is the most abundant whey protein. It constitutes nearly 60% of whey proteins. Theoretical concentrations of bovine and caprine β-lg are presented in Table 1.2. β-lg is a globular, soluble and small protein (Figure 1.4). It contains 162 amino acid residues in a single peptide chain. Its molecular mass is 18.277 kDa (Eigel et.al., 1984). pH effects its quaternary structure. It presents as a monomer (18.277 kDa) at pH 3.0 and above 8.0; octamer (140.0 kDa) at pH values between 3.5 and 5.2; and dimmer (36.7 kDa) at pH values between 5.2 and 7.0 (de Wit, 1989). The activities of β-lg are presented and summarized in Table 1.3.

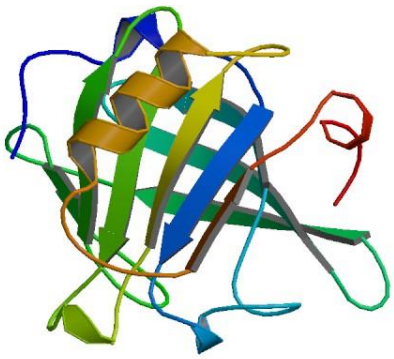


Figure 1.4. 3D structure of β-lg
(Source: Brownlow et.al., 1997)

α-lactalbumin (α-lac) is a compact globular and small protein Figure 1.5. It is secondly most abundant protein in whey. It is nearly 20% of whey proteins and composed of a single polypeptide chain. α-lac is essential physiologically for synthesis of lactose (Hernández-Ledesma et.al., 2011). It consists 123 amino acid residues and has the molecular mass of 14.2 kDa (Madureira et.al., 2007). Its structural stabilization is ensured by disulphide bonds at pH between 5.4 and 9.0 (Evans, 1982). Biological activities of α-lac presented in Table 1.3.

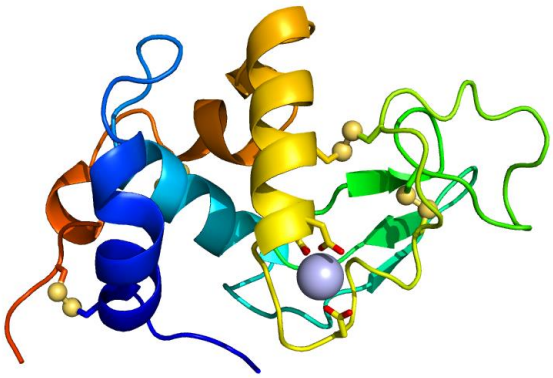


Figure 1.5. 3D structure of human α-lac
(Source: Jøhnke and Petersen, 2012)

1.3. Casein glycomacropeptide

Casein glycomacropeptide (CGMP) is the C-terminal peptide released during cheese making. Chymosin effects κ-casein; it cleaves the bond between Phe_{105}-Met_{106} and κ-casein is separated into para- κ-casein and Casein glycomacropeptide. While CGMP (Fragment 106-169) remains in whey, para- κ-casein (fragment 1-105) is insoluble and is contained within casein micelles (Martin-Diana et.al. 2002). Next to α-lac and β-lg, CGMP is the most abundant peptide/protein in whey (Bonnaillie, et.al. 2014). It comprises 12-20% of protein fractions of whey and its concentration 1.2 to 1.5 g/L in whey (Thoma-Worringer et.al., 2006).

1.3.1 Chemical Composition and Structure of Casein glycomacropeptide

Casein glycomacropeptide is the sole milk glycopeptide. Amino acid sequences of caprine and bovine CGMP (Fig 1.6) are similar, but not identical (Jollés et.al., 1961; Neeser et.al., 1988). 19 amino acid substitutions and 2 amino acid insertion are the differences between amino acid sequences of CGMP from caprine and bovine (Robitaille et.al., 2012). Mercier et al. (1976a) indicated that there is 29% differences between caprine and bovine CGMP. CGMP has not been crystallized so its 3D structure could not be certainly detectable and the existent models have been estimated (Kreuβ et.al., 2009). Bovine CGMP has 2 genetic variants, A and B frequently. These genetic variants are characterized by the differences in amino acid sequence of CGMP ($^{136}Thr \longrightarrow {}^{136}Ile$ and $^{148}Asp \longrightarrow {}^{148}Ala$) (Robitaille et.al., 2012). However, there is no genetic variation reported for caprine CGMP. Therefore, glycosylation basis characterization has been used for macropeptide from caprine whey. CGMP has two major fractions; glycosylated (gCGMP) and non-glycosylated (nCGMP) macropeptide. Both of the fractions are also phosphorylated (Kreuβ et.al., 2009). Glycosylated fraction represents about 30% of the total caprine CGMP (Robitaille et.al., 2012) and *N*-glycolylneuraminic (Figure 1.7) acid might be terminal sugar as well as *N*-acetylneuraminic acid (Figure 1.7) (Moreno et.al., 2001). CGMP has heterogeneous composition because of glycosylation. For this reason, it does not give only one peak in RP-HPLC analysis.

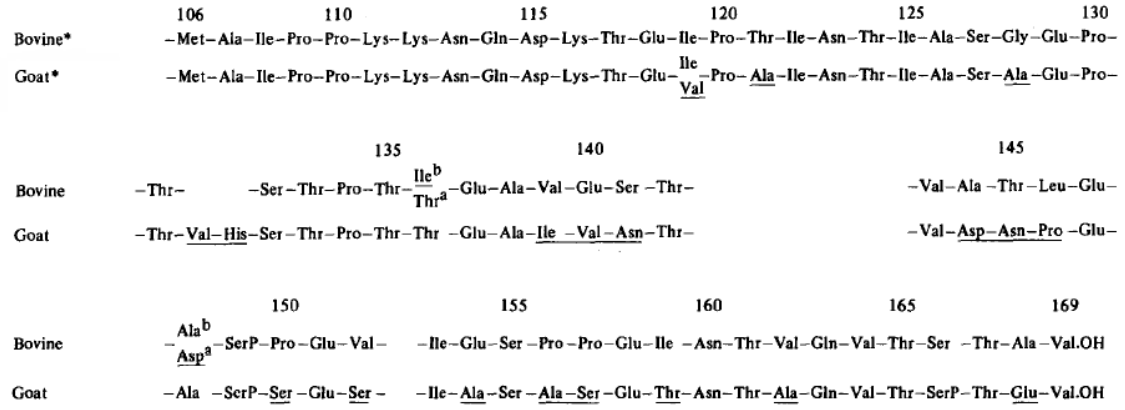


Fig 1.6. Amino acid sequences of bovine and caprine CGMP
(Source: Mercier et.al., 1976a)

Two phosphorylated serine residues ($SerP_{151}$ and $SerP_{168}$) are contained in caprine CGMP (Mercier et.al., 1976b) and nonglycosylated isoforms are mostly diphosphorylated, while CGMP from bovine whey is monophosphorylated (Robitaille et.al., 2012, Rasmussen, 1997).

Sialic acid and peptide backbone are affected functional and biological properties of CGMP (Table 1.4) (Neelima et.al., 2013). In addition, it was reported that, biological and functional features of CGMP might also be depended on its sequence of carbohydrate chain (Li and Mine, 2004).

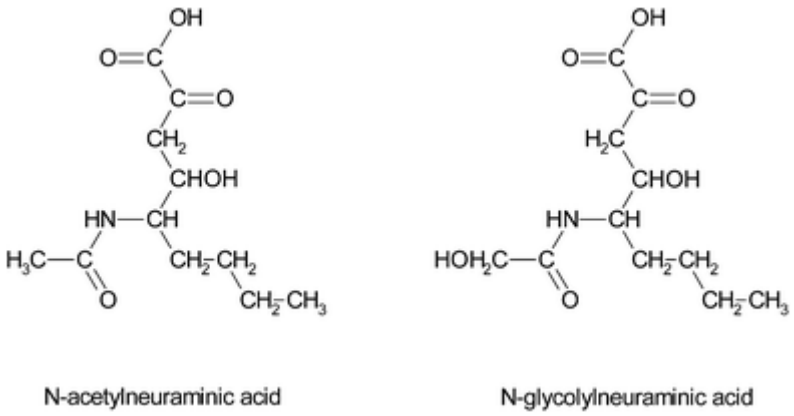


Fig 1.7. Chemical structure of *N*-glycolylneuraminic and *N*-acetylneuraminic
(Source: Alwael et.al., 2012)

Isoelectric point (pI) of CGMP is around 4.0 (Thomä et.al., 2006). However, the pI value of caprine GMP is not known precisely (Silva-Hernandez et.al., 2002). The pI value is related to acidic amino acid content of CGMP and it varies according to

phosphorylation and glycosylation of CGMP (Kreuβ et.al., 2008, Silva-Hernandez et.al., 2002; Nakano and Ozimek, 2002; Cherkaoui et.al., 1997). Kreuβ et al. (2009) found the pI values as 3.15 and 4.15 for gCGMP and nCGMP, respectively.

Molecular mass (Mr) of CGMP varies between 6.755 and 9.631. This value depends on glycosylation and pH of CGMP. On the other hand, formation of monomers to aggregates was led to specify the Mr of CGMP two to three times more than its theoretical one. Kawasaki et al. (1993b) reported the Mr of CGMP 45-50 kDa at pH>4. Farías et al. (2010) and Galindo-Amaya et al. (2006) reported that Mr of polymeric form of CGMP was ranged between 14-30 kDa. Silva-Hernandez et al. (2002) accepted the Mr of caprine CGMP as 7.5 kDa.

1.3.2 Biological Activities of Casein glycomacropeptide

CGMP contains all the phosphate and glycoside residues, which are heterogeneous and initially present in κ-casein (Eigel et.al., 1984). There is a distinguishable differences in non-peptide fragments of species such as ewe, goat or cow (Jollés, 1961) which are probably the cause of differences in the biological and functional behavior of species. For these reasons the functional and biological properties of caprine whey proteins, might differ from the functional and biological features of bovine whey proteins. In the literature there is a lack of information on the functional properties of the CGMP obtained from caprine whey. Therefore, Table 1.4 includes the functional and biological features of bovine CGMP mostly, which reported in literature, are summarized below and the effects of the differences on the biological features are presented in Table 1.4.

Vibrio cholera produces Cholera toxin. This toxin binds to gangliosides and then causes gastro enteric disorders which causes possible deaths (Madureira, et.al., 2007; Kawasaki et.al., 1992). Glycosylated CGMP can inhibit the binding of Cholera toxin to gangliosides and can prevent gastro enteric disorders. When CGMP was treated with sialidase, inhibition activity was reduced rapidly (Oh et.al., 2000). Sialidase hydrolyses the sialic acids and this might be suggested the significance of sialic acid for the binding activity of CGMP.

Table 1.4. Status of peptide backbone and carbohydrate chain in various biological features of CGMP

Biological Features	Effect of carbohydrate chain	Effect of peptide backbone	References
Binging of Cholera toxin	x		Kawasaki et.al., 1992
Inhibition of hemagglutination	x		Kawasaki et.al., 1993a
Intestinal infection prevention	x		Nakajima et.al., 2005
Nutritional management of PKU		x	LaClair et.al., 2009; van Calcar and Ney, 2012
Anti-cariogenic effect		x	Neeser et.al., 1988, 1994; Aimutis, 2004
Antibacterial activity		x	Robitaille et.al., 2012
Immunomodulatory activity	x	x	Otani et.al., 1992; Requena et.al., 2010
Growth promoting effect on Bifidobacter		x	Azuma et.al., 1984
Reduction of gastric secretion		x	Aleinik et.al. 1984

The acidity of stomach prevents the growth of pathogens (Martinsen et.al., 2005). Its ambient pH is about 2.0 and this pH inhibits the *Escherichia coli* rapidly. However, ambient pH of stomach can be increased after food intake and stomach becomes mostly suitable for growth of *E.coli* (Zhu et.al., 2006). CGMP is capable to inhibit heat labile enterotoxins of *E.coli* and prevents the diarrhea that the toxins cause (Isoda et.al., 1999).

Actinomyces viscosus, Streptococcus sanguis, S. mutans, and *S. sobrinus* that are cariogenic bacteria adhere on dental surface. CGMP prevents haemagglutination by *A. viscosus, S. mutans* and *S. sanguis*. In this way, it inhibits the adhesion of these species on dental surface. It can also prevent the formation of dental caries and dental plaque and maintain oral health (Neeser, 1987; Neeser et.al., 1988).

It was reported that CGMP can protect cells against infection. Influenza virus infect the cells and CGMP inhibits virus heamagglutination with sialic acid (Kawasaki et al, 1993).

CGMP is an antihypertensive peptide. It has angiotensin converting enzyme (ACE)-inhibitory features. It was found that, undigested caprine CGMP indicated ACE-inhibitory activity moderately, but digestion under gastrointestinal conditions was increased the inhibitory activity considerably and then effective antihypertensive tri-peptide was secreted. Consequently, this feature of CGMP can be used in the hypertension treatment (Miguel et.al., 2007).

Inflammatory response has a step as splenocyte proliferation. Suppression of immune response is shown by inhibition of splenocyte. Otani et al. (1992) reported that lipopolysaccharide of mitogen *Salmonella typhimurium* induces splenocyte proliferation of mouse and casein can inhibit this reaction. Inhibitory activity is based on kappa-casein. Para-kappa-casein does not show inhibitory activity; CGMP has this activity. Sialic acid is crucial for this feature. When sialic acid hydrolyzed by sialidase, CGMP lost its activity (Madureira et.al., 2007).

CGMP demonstrated a growth-promoting effect on both *Lactococcus* (Bouhallab, et.al., 1993) and *Bifidobacteria* species (Idota et.al., 1994) and glycosylated *N*-acetylneuraminic acid attributed to this prebiotic activity (Bouhallab et al. 1993).

Guilloteau et al. (2010) showed that CGMP can decrease gastric secretions stimulated by the food, but there was dose-independent response. Besides, Chernikov et al. (1974) reported that the presence of CGMP into a part of small intestine significantly decreased gastric secretion. In another study, Aleinik et al. (1984) reported that existence of CGMP reduced acid secretion in rat stomachs by 53% and reduced gastrin content of blood serum by 8.3%. That study also reported that, peptide hormones which promote the yield of milk protein digestion and formed in the gastric mucosa are responsible for inhibition of gastric secretion by CGMP.

Xu et al. (2013) reported that CGMP can decrease lipid accumulation and promote antioxidant capability of obese rats. This shows that CGMP can counteract high-fat diet-induced obesity, and this situation might make CGMP a possible ingredient with anti-obesity activity.

1.3.3 Isolation Processes of Casein glycomacropeptide

Several methods have been described for large-scale preparation of Casein glycomacropeptide (CGMP). A method for the preparation of CGMP used a special ion exchange medium called Spherosil QMA (Skudder, 1983, 1985). This medium consists of highly porous silica spheres to which a copolymer of styrene-vinyl triethoxysilane carrying a strong base group of -$N(CH_3)_3Cl$- is grafted. When whey was passed through this medium at pH 5.0, the CGMP was selectively adsorbed and even displaced the whey proteins initially adsorbed on the column. The adsorbed CGMP can then be recovered from the medium by elution with dilute HCI or NaCl solutions. This method has been covered by a patent (Burton & Skudder, 1987). Spherosil has the advantage that it is non-biodegradable and does not swell or contract with changes in ionic environment.

CGMP was also obtained industrially by pepsin or rennet digestion of sodium caseinate using a deproteinized and delactosed whey of rennet casein curd, the pH of which was lowered to precipitate calcium phosphate, followed by concentration by reverse osmosis and freeze-drying (Dosako et.al., 1988).

Tanimoto et al. (1990) described a method for producing κ-casein CGMP based on the pH-dependent molecular weight of CGMP. The pH of the whey was adjusted to <4, where CGMP was largely in its monomeric form (mol. wt 8000), and then ultrafiltered. Permeate contained the CGMP while other whey proteins were retained under these conditions. Permeate was then adjusted to pH 7.0 where CGMP forms aggregates of mol. wt > 32,000. The solution was then ultrafiltered again to concentrate the CGMP.

Saito et al. (1991) heated a solution (10%, v/v) of reconstituted whey powder at pH 6.0 and 98°C for 1 h, followed by precipitation with cold ethyl alcohol (50%, v/v). The precipitate was separated by centrifugation and the supernatant was adjusted to pH 9.0, and subjected to chromatography on a DEAEToyoperal 650 M column. The adsorbed material was then eluted with 0.3 M ammonium bicarbonate and evaporated to dryness under vacuum. The residue was dissolved in water, insoluble material was removed by centrifugation and the solution was freeze dried. A yield of 1.18 g CGMP from 100 g whey powder was reported.

Kawasaki and Dosako (1992) described a process for the production of κ-casein CGMP. The method involved an ion exchange treatment of whey, where the CGMP was

not adsorbed on the ion exchange column under the used conditions. The fraction containing the CGMP was then concentrated, desalted and dried.

Martin-Diana et al. (2002) have developed a method for isolating CGMP from whey that based on the thermostability of casein glycomacropeptide (CGMP) and on the differences in molecular weight of its polymeric and monomeric forms. Whey, obtained by rennet clotting of milk, was adjusted to pH 3.5 with 0,1 N HCl, heated to 90˚C for 1 h and centrifuged (5200g, 4℃, 15 min) to remove the denatured proteins. The supernatant was brought to neutral pH with 0.1 mol/l NaOH with 0.1 mol/l NaOH under vigorous stirring and ultrafiltered at 50℃ with a 10 kDa UF membrane (Millipore Pellicon Filtres). Retentate, which contained the CGMP, was diafiltered to eliminate the excess of salts and lactose, concentrated and finally lyophilized. This yielded a product of concentration 58–75% CGMP.

Casein glycomacropeptide (CGMP) was purified from Chymosin-hydrolyzed caseinate solution. The procedure involving gel chromatography on Sephacryl S-200 at pH 7.0 to obtain a crude CGMP fraction, followed by precipitation of contaminating protein and/or peptide with addition of acidic solution, pH 3.5 to the crude glycomacropeptide. Supernatant, which was soluble in the acidic solution, was subjected to chromatography again on Sephacryl S-200 at pH 3.5. The purified CGMP accounted for 5.3% of dry weight of caseinate hydrolysate, and 0.7% of dry weight of sodium caseinate powder. The preparation was of considerably high purity with its amino-acid composition showing only traces (each < 1 residue/peptide) of arginine, histidine, phenylalanine, and tyrosine, the amino acids that do not occur in CGMP (Nakano and Ozimek, 2002).

1.3.4. Functional Properties of Casein glycomacropeptide

Gel formation, foaming, and emulsification properties are the functional properties of CGMP. Glycosylation of CGMP affects the functional properties as it affects the biological properties. In literature, any studies were found about functional properties especially for caprine CGMP. For this reason, studies with bovine CGMP were summarized in this section.

1.3.4.1. Foaming and Emulsification Properties

Foaming and emulsification properties are foremost functional features of milk proteins. When compared with whey protein concentrate, CGMP was shown lower emulsifying activity index (185 m^2/g for whey protein concentrate and 36 m^2/g for CGMP) (Neelima et.al., 2013), but the stability of emulsifying activity index for CGMP was higher than whey protein concentrates. CGMP shows emulsifying properties in the large pH range, so this might be an advantage for food processing. Glycosylation status influence the emulsification features. Emulsifying activity index for nCGMP was higher than gCGMP (150.7 m^2/g for nCGMP and 98.5 m^2/g for gCGMP). nCGMP also had higher stability of emulsions as compared to gCGMP.

Foaming feature also relates with glycosylation. A mix with 10% CGMP content had higher overrun, but it shown lower foam stability than mix contained egg white. It was revealed that CGMP added fruit gels (carrageenan was also added as gelling agent) were had an improved gel firmness and better appearance at pH 4.5 than fruit gels produced with no CGMP (Marshall, 1991).

1.3.4.2. Gel-Forming Properties

Burton and Skudder (1987) studied on gel formation features of CGMP. They found that, a solution involving 9.3% CGMP at 20˚C and pH 4.5 could form a gel, but not heat treated at 90 ˚C. In another study, Marshall (1991) did not have the same results. In addition to this, Ahmed and Ramaswamy (2003) reported that a solution involving 12.5% CGMP at pH 7.0 at 80˚C formed a gel. Martin-Diana et al. (2004) investigated the rheological features of fermented caprine milk with addition of CGMP and found that CGMP was decreased the phase angle, improved the elasticity, and structured more regular gel than gel produced with whey proteins.

1.3.5. Detection Methods of Casein glycomacropeptide

Various methods have been reported for detection of CGMP. The methods used for detection of CGMP are summarized in this section. The presented methods used for detection CGMP from both bovine and caprine whey.

1.3.5.1 Chromatographic Methods

Chromatographic separation of CGMP from other whey proteins are presented. CGMP is characterized by the lack of aromatic amino acids. Therefore it is detectable only between 205-217 nm and it is invisible at 280 nm (Abd El-Salam et.al., 1996). For these reasons CGMP is detected at absorbance value between 215-217 nm.

1.3.5.1.1. Size Exclusion Chromatography

Van Hooydonk and Olieman (1982) reported a HPLC method depends on precipitation of other whey proteins except CGMP by using Trichloroacetic acid (TCA). TSK 2000SW (30cm x 0.75cm internal diameter) was used. CGMP was detected quantitatively at 35˚C. 12% TCA was used to precipitate other whey proteins. The eluent is composed of a solution that contained Na_2SO_4 (21.41 g), KH_2PO_4 (12.37 g), and K_2HPO_4 (1.74 g) in 1000 ml double distilled water and filtered through 0.45 μm filter. Subsequently the eluent was kept at 85˚C in order to inhibit bacterial growing and to retain it degassed. The analysis was completed in 40 min and a single peak could be obtained for CGMP. This method does not give data/peak for glycosylated or non-glycosylated fraction of CGMP specifically.

Kawakami et al. (1992) determined the CGMP by anion exchange chromatography using Mono Q HR 5/5 column and size-exclusion chromatography equipped with two coupled TSK gel G3000 PW columns. Firstly, CGMP was separated by size exclusion chromatography. Solvent was consisted of 55% (v/v) acetonitrile in 0.1% trifluoroacetic acid (TFA). Chromatogram consisted of a single peak for CGMP. Following this analysis, anion exchange chromatography was used to examine the separated CGMP. Nine peaks were obtained for CGMP fraction, seven of them were glycosylated CGMP and two came from other casein sources. Samples were not pretreated with TCA for this analysis. Detection of CGMP fractions and determination of CGMP content without pre-treatment of samples with TCA were supremacy of this described method.

A modified HPLC method, described by Sharma et al. (1993), was used to determine CGMP. Size exclusion chromatography was equipped with just one gel permeation column (Protein Pak-125, 30 cm x 0.78 cm). Same eluent was used as Van

Hooydonk and Olieman (1982) and it was at room temperature. This method was run for 125 min. CGMP was eluted as aggregated monomeric CGMP in this method.

1.3.5.1.2 Ion Exchange Chromatography

CGMP was separated by using cation exchange chromatography and fast liquid chromatography together equipped with mono S column (Léonil and Mollé, 1991). Firstly Casein was precipitated from the whey at pH 4.6. Supernatant was fed into the chromatographic column directly. 8% TCA solution was used to precipitate milk protein, and 50-75% of the CGMP could be recovered in the supernatant. The eluent was composed of 0.1 M NaCl in 20 mM KCl-HCl buffer, at pH 2.0. The CGMP was obtained as a single peak. RP-HPLC was also used to reveal genetic variants (A and B) of separated CGMP with a Vydac column.

1.3.5.1.3. Reversed Phase Chromatography (RP-HPLC)

Various RP-HPLC methods have been reported. It appeared that RP-HPLC methods are most widely used detection methods for CGMP because genetic variants and glycosylated fractions of CGMP can be detected by RP-HPLC. Some of methods were presented in Table 1.5.

1.3.5.2. SDS-PAGE Analysis

CGMP has been detected by a method based on SDS-PAGE developed by Laemmli (1970). Electrophoresis was run with a 4% stacking gel and 12% separating gel at 100 V for 100 minutes. Protein staining was performed by Comassie Brilliant Blue R250 for 30 min and destaining was carried out for 2 h. Molecular mass of CGMP is influenced from glycosylation and pH. For this reason, samples must necessarily be precipitated with TCA in order to detect by this method.

3.5.3. Capillary Electrophoresis

In this technique, molecules are detected based on their electrophoretic mobility. Whey proteins have separated by a capillary electrophoretic method described by Otte et al. (1995) under acidic and basic conditions. A CGMP peak was separated and detected from the other whey proteins under acidic conditions (70 mM phosphate, pH 2.5). In another study, genetic variants (A and B) of CGMP were separated and detected by using capillary electrophoresis (Van Riel and Olieman, 1995).

1.3.5.4. Colorimetric Methods

CGMP are includes all the *N*-acetylneuraminic acids (sialic acids) of casein. Thereby CGMP can be measured by determining the sialic acid in the Trichloroacetic acid (TCA). In a method described by Warren (1959), firstly samples were hydrolyzed with acid in order to release the *N*-acetylneuraminic acids and then these *N*-acetylneuraminic acids were determined by the thiobarbituric acid reaction. This determination method gives an approximate quantities about CGMP content, so this method can be used for comparison of CGMP contents in different studies. On the other hand, caprine CGMP contains *N*-glycolylneuraminic in addition to *N*-acetylneuraminic as terminal sugar. For this reason, sialic acid detection is not an exact method for CGMP from caprine whey.

Table 1.5. Different RP-HPLC based detection methods of CGMP (ACN: acetonitrile, TFA: trifluoroacetic acid)

Reference	Column	Mobile Phase A	Mobile Phase B	Flow Rate (ml/min)	Temperature(˚C)	Absorbance (nm)	Detector
Saito et.al., 1991	C18, Asahipak ODP-50, (6.0 x 250mm)	0.05% TFA in ACN:water(10:90)	0.05% TFA in ACN:water(60:40)	0.5	50	210	Uv detector
Martin-Diana et.al., 2002	C18 Nucleosil column (300Å, 250 x 4.6mm, 5µm particle size)	0.1% TFA in water	0.1% TFA in ACN	1.0	40	214	Uv detector
Bonnaillie et.al., 2014	C4 with polystyrene-divinylbenzene column (250 x 4.6mm, 3.5µm particle size)	0.1% TFA in ACN	0.1% TFA in water	0.8	30	214	Uv detector
Sanmartin et.al., 2012	Source 15RPC column (4.6 x 100mm)	0.1% TFA in water	0.09% TFA in ACN:water(90:10)	1.0	25	214	Uv detector
Thoma et.al., 2006	PLRP-S 300Å 8mm column	0.1% TFA in water	0.0555% TFA in ACN:water(80:20)	1.0	40	226	Uv detector
Holland et.al., 2010	C2/C18 column with guard column (4.6 x 100mm)	0.1% TFA in water	0.1% TFA in ACN:water(90:10)	1.0	Not given	210	Uv detector
Coolbear et.al., 1996a	C18 Synchropak HP-P column (4x240mm)	HCOOH (0.5ml/l), NaCl (10g/l), ACN (100ml/l) in water	HCOOH (0.5ml/l), NaCl (10g/l), ACN (600ml/l) in water	1.0	20	Not given	Not given
Molle and Leonil, 2005	C18 narrow-bore symmetry column (2.1 x 150mm, 5µm particle size) with C18 cartridge guard	0.106% TFA in water	0.1% TFA in ACN:water(80:20)	0.25	40	Not given	Not given

1.3.6. Nutritional Importance of Casein glycomacropeptide

The CGMP has unique amino acid composition. It can be used as food ingredient and supplement. CGMP lacks all aromatic amino acids (Phe, Tyr, and Try) Thanks to this features; it can be used in the special formulation for consumption of individuals with PKU. (Smithers et.al., 1991). CGMP is rich in branched chain amino acids (valine and isoleucine) and poor in methionine. This features make CGMP useable in dietary of Hepatitis patients. However all these features are dependent of the similarity of amino acid compositions that of CGMP from κ-casein and from whey. Shammet et al. (1992) reported that Chymosin might act differently to CGMP obtained from whole casein and developed from κ-casein.

Special foods developed for these individuals are produced by using synthetic amino acid mixtures. These are lack of functional properties and palatability of proteins. Hence, the use of CGMP in food developments would improve the flavor and proper mouthfeel of proteins to these foods.

The objective of this thesis is to extent the product range of PKU diets and contribute the knowledge about Casein glycomacropeptide and malnutrition. Within this context, CGMP was isolated from sweet caprine whey. Therefore this study not only contributes to develop a nutritional food ingredient but also to the solution of environmental pollution problems caused by whey disposal.

CHAPTER 2

MATERIALS and METHODS

2.1 Materials

Whey for ultrafiltration process was kindly provided by Türer A.Ş. Rennet for cheese-making was kindly provided from Intermak A.Ş. and Maysa A.Ş.

2.2. Methods

2.2.1. Isolation Methods of Casein glycomacropeptide

2.2.1.1 Ultrafiltration

Isolation of CGMP by using an ultrafiltration system (Sartorius AG, Goettingen, Germany) was followed by a procedures described by Martin-Diana et al. (2002). The cheese whey obtained from a local cheese producers were used directly. Initial pH of whey was 5.6. In the Experiment 1 (Figure 2.1), the pH of whey was previously adjusted to 3.2 with 0.1 N HCl. Then, the whey was heated and kept at 80°C for 1 h to utilize the thermo-resistance of CGMP (Saito, et al, 1991). The heat treated whey was cooled to 4°C and kept overnight. The next day, centrifugation was carried out at 5200 x g, at 4°C for 15 min to remove the denatured protein components. After centrifugation the pH of supernatant was adjusted to neutral pH with 0.1 M NaOH and then ultrafiltration was performed using a Hydrosart membrane with a MW cut off of 10 kDa (Sartorius AG, Goettingen, Germany). The retentate containing the CGMP was then lyophilized and stored at -20°C until analysis.

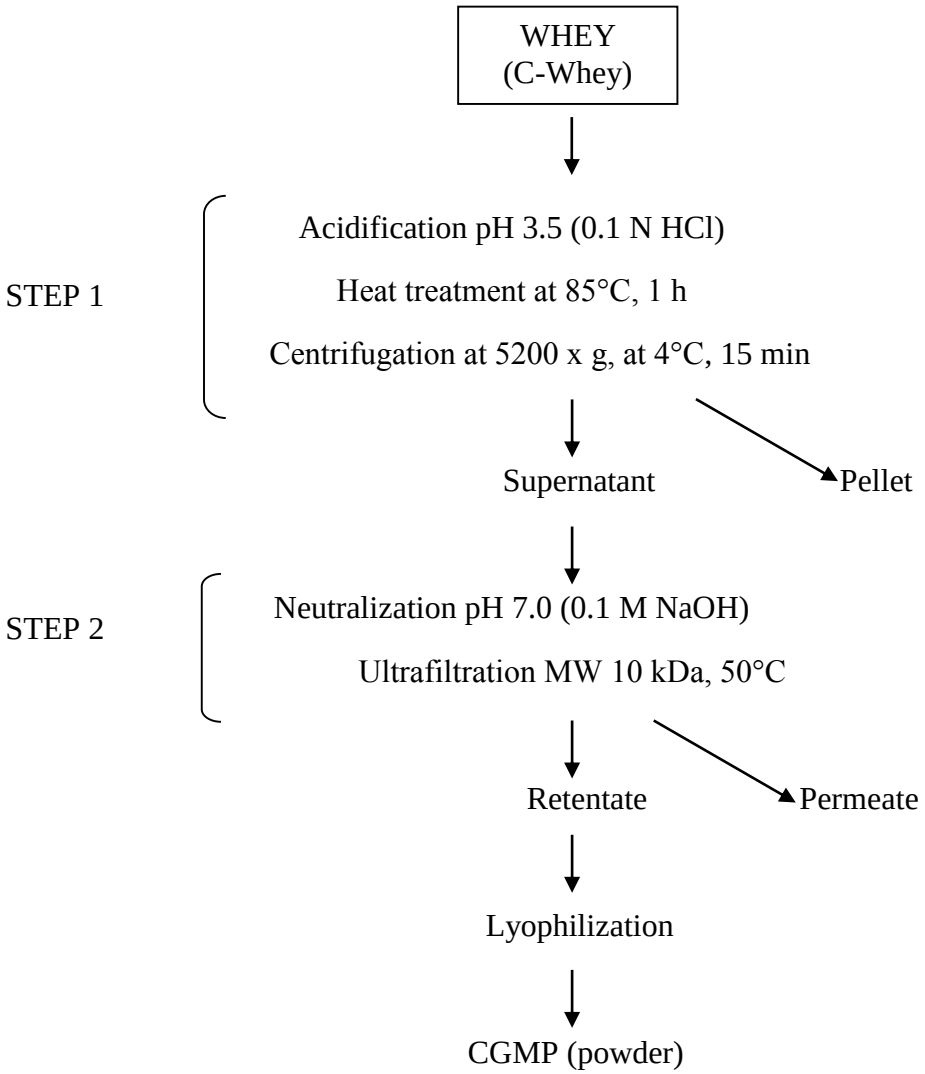


Figure 2.1. Experiment 1: Isolation procedure diagram of CGMP
(Source: Martin-Diana et.al., 2002)

In Experiment 2 (Figure 2.2), a modified procedure of Martin-Diana et al. (2002) that described by Karakaya (2012) was used because of the high protein and lactose content of Retentate in Experiment 1. Neutralization process were removed. Parameters of heat treatment and centrifugation were altered for more effective protein isolation. Heat treatment was performed at 95°C for 120 min and centrifugation was carried out at 10000 x g for 20 min. Step 3 was added in order to reduce the overdose of lactose. In this diafiltration step, firstly volume of Retentate 1 was completed to initial volume of whey with distilled-water and then again filtered. The Retentate 2 containing the CGMP was then lyophilized and stored at -20°C until analysis.

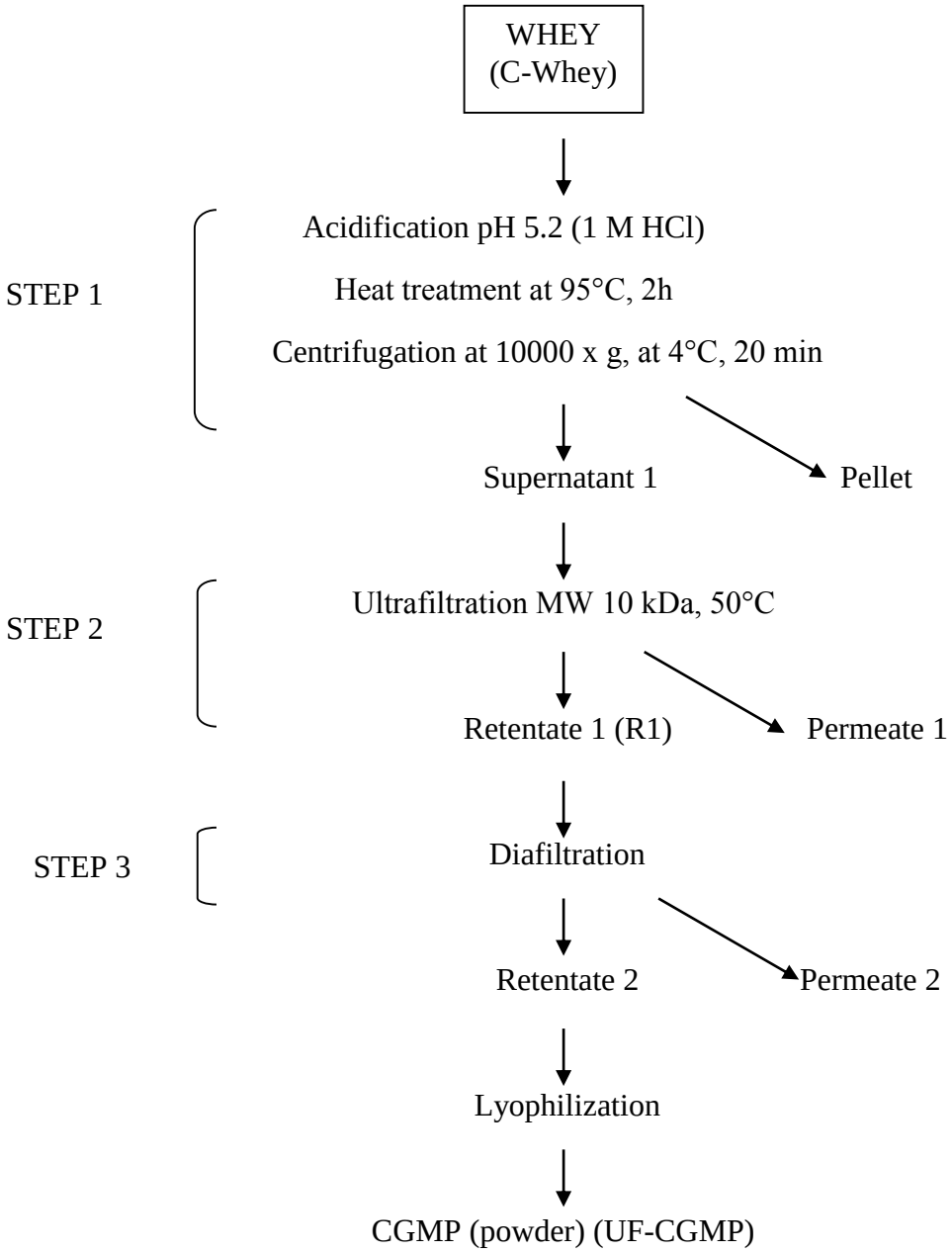


Figure 2.2. Experiment 2: Isolation procedure diagram of CGMP
(Source: Karakaya, 2012)

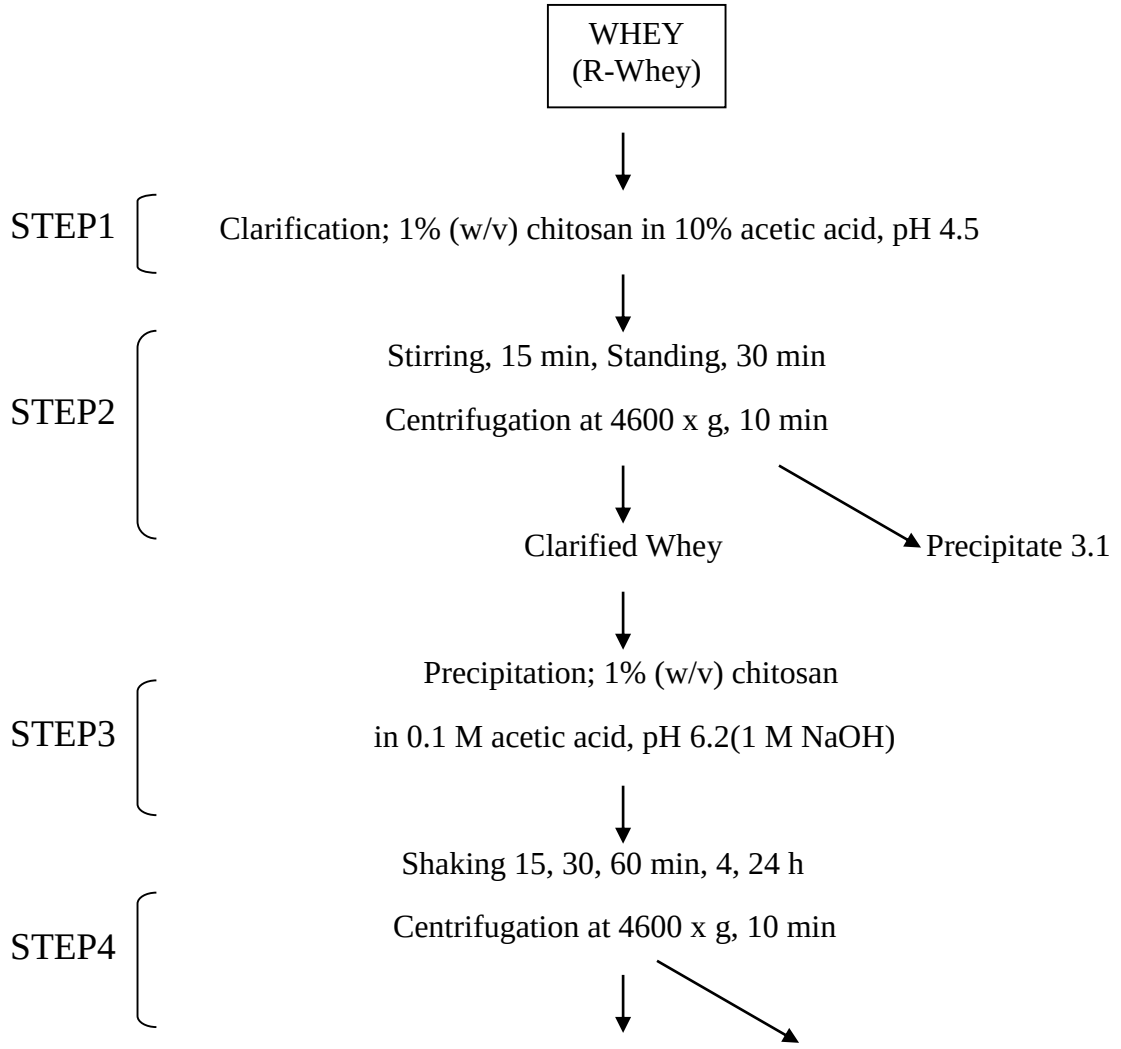
2.2.1.2. Chitosan Treatment

During heat treatment, whey proteins except CGMP were precipitated. These whey proteins have various functional and nutritional features. Especially, β-lg has substantial functional properties for food industry. Therefore, β-lg was attempted to be isolated without denaturizing before heat treatment. A method described by Casal et al. (2006) was modified and used for this purpose. In this method (Fıgure 2.3) (Experiment

3), firstly 1% (wt/vol) chitosan in 10% acetic acid was added to whey to obtain solution of 0.25 mg of chitosan/ml for precipitation of lipids and suspended solids. The pH of whey was decreased from 6.04 to 4.5 after addition of chitosan-acetic acid solution. The mixture was vigorously stirred for 15 min and allowed to stand for 30 min and centrifuged at 4600 x g for 10 min.

A second treatment with chitosan was performed to recover the β-lg. The chitosan concentration of clarified whey, obtained during the first precipitation, was adjusted to 1.4 mg chitosan/ml with 1% (wt/vol) chitosan in 0.1 M acetic acid and the pH was increased to 6.2 with 1 M NaOH. The mixture was shaken in a shaking incubator at 75 rpm, at 25°C for 24 h, and then centrifugation was performed at 4600 x g for 10 min.

Isolation of CGMP from Supernatant 3.1 was performed according to the method considered to be most effective. The supernatant 3.1 considering CGMP was heat treated at 95°C for 120 min and centrifuged at 10000 x g, at 4°C min, for 20 min. This Supernatant 3.2 was lyophilized without ultrafiltration and stored at -20°C until analysis.



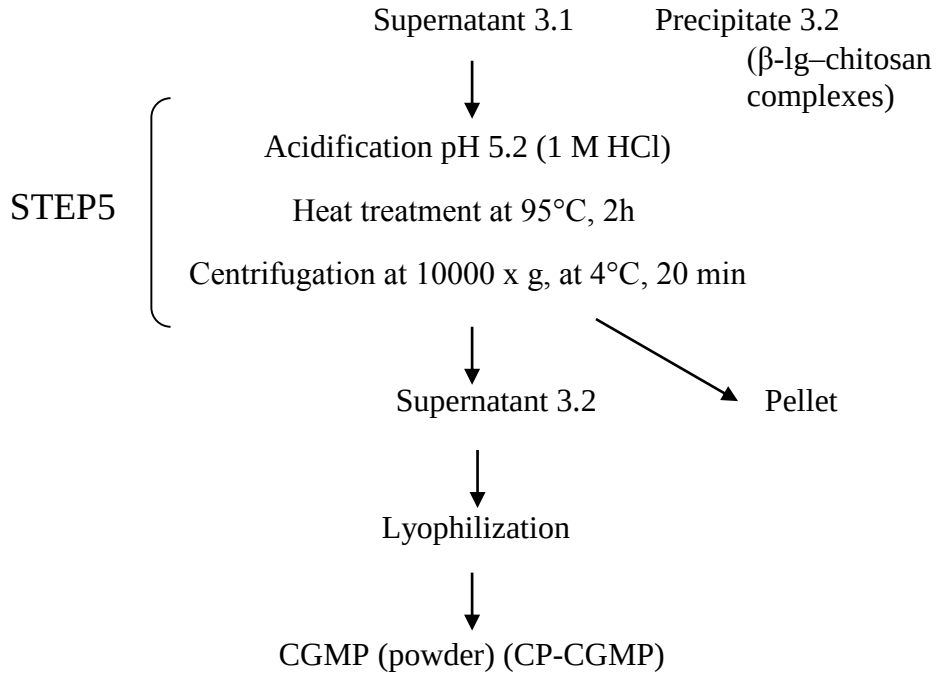


Figure 2.3. Experiment 3: Isolation procedure diagram of CGMP by chitosan treatment

In order to determine the effects of chitosan on isolation process of CGMP, Experiment 4 was carried out (Figure 2.4). In this experiment, the same whey was used with Experiment 3 and acidification and heat treatment was applied without chitosan precipitation.

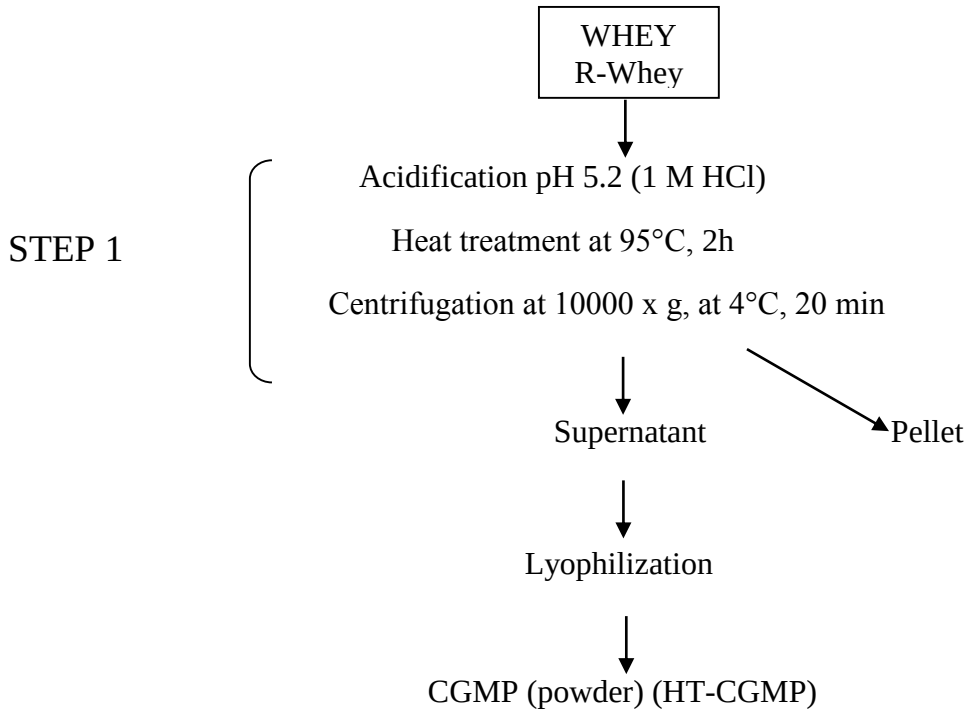


Figure 2.4. Experiment 4: Isolation of CGMP without ultrafiltration process

2.2.2 Preparation of Rennet Whey

Fresh goat's milk (unpasteurized) was warmed and when the temperature reached to 37°C, 3.7 ml rennet solution added. After coagulation, the curd and whey was gently stirred. Finally, the whey was filtered through cheesecloth and stored at -20°C until analysis.

2.2.3. Protein Fraction Analysis

2.2.3.1. SDS-PAGE Analysis

Proteins in the samples were determined using SDS-PAGE according to Laemmli (1970). Undiluted samples were mixed with sample buffer (1:1) and boiled for 5 minutes to denature proteins. Electrophoresis was run with a 4% stacking gel and 12% separating gel at 100V for 100 minutes. The volume of protein sample loaded to each well was 20µl. Calibrated α-lac, β-lgA and β-lgB standards were used in three of the gel lanes. Protein staining was performed by Comassie Brilliant Blue R250 for 30 min and destaining was carried out for 2 hours. The protein composition of the fractions were analyzed by VersaDoc gel documentation system (Bio-Rad, Hercules, CA, USA). The system was corrected according to the calibration sample.

2.2.3.2. HPLC Analysis of Whey Proteins

Chromatographic analysis of whey proteins (α-La, β-Lg, CGMP) was carried out by reverse-phase (RP) HPLC on a Perkin-Elmer Series 200 HPLC (USA) chromatograph (Table 2.1). An Agilent ACE C18 column (150x4.6 mm, 5-µm particle size, Agilent Technologies, Spain) was used. The chromatograph was equipped with a Perkin-Elmer UV detector which was set to 214 nm. The mobile phase A was 0.1% trifluoroacetic acid (v/v) in double distilled water and B was 0.1% (v/v) trifluoroacetic acid in acetonitril-water (80:20). Analysis was conducted at a linear gradient program. The gradient of B was increased from 0 to 100% in 40 min and then returned to starting conditions in 5 min. The flow rate was 1 ml/min and the temperature was 40°C using 20 µl fractions. The fractions were analyzed directly, without dilution. α-La and β-Lg standards were prepared at different concentrations (0.25 mg/ml – 3 mg/ml) in order to plot the standard calibration

curves. Mobile phase A was used for dilutions. All the fractions and standard solutions were syringe filtered through 0.45 μm filters. Peak areas of standard solutions, whey proteins and CGMP were used for quantification. The results were expressed as percentage peak areas in the chromatograms for CGMP and mg/ml of isolate for α-La, β-Lg.

Table 2.1. RP-HPLC conditions used for the chromatographic analysis of whey proteins.

RP-HPLC	DAD detector
Column	ACE C18 column (150x4.6mm, 5-μm, 300Å)
Mobile Phase A	0.1% trifluoroacetic acid (v/v) in double distilled water
Mobile Phase B	0.1% (v/v) trifluoroacetic acid in acetonitril-water (80:20)
Flow Rate	1ml/min
Temperature	40°C
Wavelength	214 nm
Injection volume	20μl

2.2.3.3. Purity Control of Target Fraction

The purity of CGMP fraction was determined from the ratio of their peak areas to the total peak area of all peaks obtained by RP-HPLC.

2.2.4. Phenylalanine and Tyrosine Analysis

2.2.4.1. Phenylalanine and Tyrosine Detection by RP-HPLC

Phenylalanine is an essential amino acid and it cannot be synthesized by the metabolism. Namely, it must be taken from foods. Phenylketonurics who cannot metabolize phenylalanine must maintain inflexible phenylalanine-restricted diet. Thereby, tyrosine cannot be sufficiently derived from phenylalanine and becomes essential for metabolism. For all these reasons, phenylalanine and tyrosine contents of

developed ingredient should be known. Phenylalanine and Tyrosine analysis were carried out by reversed–phase high performance liquid chromatography (RP-HPLC) using a ACE 5 C18 column (300Å, 250 x 4.6 mm i.d., 5µm particle size; Advanced Chromatography Technologies, Aberdeen, Scotland) at a flow rate 0.8 ml/min, at 25°C. Firstly, fractions were hydrolyzed in a condenser at 100°C for 24 h in 6 M HCl (100 ml HCl / 1 g sample) (Table 2.2). 0.1% (w/v) phenol solution was added in order to inhibit oxidation. HCl in hydrolyzed fractions was evaporated with a rotary vacuum evaporator at 65°C. After the evaporation of HCl, hydrolyzed fractions were cooled to room temperature and 1 ml mobile phase was added. All the samples were syringe filtered through 0.20 µm filters before RP-HPLC analysis. 0.0125 M KH_2PO_4 : acetonitrile (98:2) solution was used as mobile phase (Prodolliet and Bruelhart, 1993). The pH of phosphate buffer solution was adjusted to 3.5 with 5% phosphoric acid solution. Absorbance was recorded at 214 nm using a Perkin –Elmer DAD detector (Perkin-Elmer, Massachusetts, USA). Phe and Tyr standards were prepared at different concentrations (0.01 mg/ml – 0.15 mg/ml) in order to graph the standard calibration curves. Phe and Tyr standards were diluted with distilled water and 0.1 N HCl respectively. Contents of phenylalanine and tyrosine in whey and lyophilized CGMP isolate were expressed as the weight basis (w/w). The identity of amino acids was confirmed by comparison of their retention times with those of the Phe and Tyr standards purchased from Merck (Kenilworth, USA).

Table 2.2. RP-HPLC conditions used for the chromatographic analysis of phenylalanine and tyrosine.

RP-HPLC	DAD detector
Column	ACE C18 column (150x4.6 mm, 5-µm, 300Å)
Mobile Phase A	0.0125 M KH_2PO_4 : acetonitrile (98:2)
Flow Rate	0.8 ml/min
Temperature	25°C
Wavelength	214 nm
Injection volume	20µl

2.2.5. Compositional Analysis

2.2.5.1. Dry Matter

Dry matter content of samples was expressed as % of initial sample test portion weight according to Eq 2.1 (AOAC, 2006). Test portions were dried at 105°C in an oven until constant weight.

$$\text{Dry matter, } \% = \frac{(W_2\text{-}W)}{(W_1\text{-}W)} \text{ x } 100 \qquad \text{Eq 2.1.}$$

where, W is weight of dish; W_1 = weight of dish + sample test portion; W_2 = weight of dish + dry sample.

2.2.5.2. Ash Content

Samples (3 g) were prepared into predried crucible, and evaporate to dryness in furnace. The prepared test portions were incinerated in furnace at 550°C for 6 h. The crucibles were removed from furnace, cooled to room temperature in desiccators, and weighed. %, ash content of samples calculated according to Eq 2.2 (AOAC, 2006).

$$\text{Ash Content, } \% = \frac{(W_2\text{-}W)}{(W_1\text{-}W)} \text{ x } 100 \qquad \text{Eq 2.2.}$$

where, W is weight of crucible; W_1 = weight of crucible + sample test portion; W_2 = weight of crucible + dry sample.

2.2.5.3. Total Protein and Non-protein Nitrogen Determination

Saito et al. (1991) reported that non-protein nitrogen was the sole source of CGMP and total whey protein content can be determined by subtracting non-protein nitrogen content from total protein content. Therefore, total protein and non-protein nitrogen contents of samples were determined by Kjeldahl method using a conversion factor of 6.38 (AOAC, 2006). Vapodest 50s; a software controlled and equipped with integrated titration module, semi-automatic system (Gerhardt GmbH & Co., Bonn, Germany) was used for these determinations. For detection of total protein content, 1 g of samples were weighed in digestion tubes. 3.5 g K_2SO_4, 1 ml $CuSO_4.5H_2O$ and 15 ml H_2SO_4 were also

added to digestion tubes in order to release nitrogen from protein and retain nitrogen as ammonium salt. The samples in the tubes were digested at 420°C for 4 h in block digestion unit. After the digestion process samples were diluted and 32% NaOH was added to release NH_3, collected in H_3BO_3, and titrated with 0.1 N HCl. Eq 2.3 and Eq 2.4 was used, respectively to calculate protein content.

$$\text{Nitrogen, \%} = \frac{1{,}4007 \text{ x Vc x M}}{\text{W}} \qquad \text{Eq 2.3}$$

Where, M: molarity of HCl solution; Vc: HCl consumption for test portion during titration; and W: test portion weight, g. Percent nitrogen was multiplied by 6.38 to calculate percent protein.

$$\text{Protein content, \%} = \text{Nitrogen, \%} \times 6.38 \qquad \text{Eq 2.4}$$

Non-protein nitrogen content was determined by precipitation of protein from samples with Trichloroacetic acid (TCA). 10 ml sample was poured into preweighed flask and weighed. Then 40 ml, 15 % (w/v) TCA concentration, was added to flask and again weighted. Precipitated whey proteins were removed by filtration through Whatman No. 1 paper filter. Non protein nitrogen components were remained in filtrates. 20 ml of filtrates was weighed with beaker and then poured to Kjeldahl flask and empty beaker was weighted. All weights were recorded. 3.5 g K_2SO_4, 1 ml $CuSO_4.5H_2O$ and 15 ml H_2SO_4 were also added to digestion tubes. The filtrates in the tubes were digested at 420°C for 4 h in block digestion unit. After the digestion process samples were diluted and 32% NaOH was added to release NH_3, collected in H_3BO_3, and titrated with 0.1 N HCl. Eq 2.5 and Eq 2.6 was used respectively to calculate non-protein nitrogen content.

$$\text{Nitrogen, \%} = \frac{1{,}4007 \text{ x Vs x M}}{(\text{Wf x Wm})/[\text{Wt}-(\text{Wm x a})]} \qquad \text{Eq 2.5}$$

Where, V_s : consumption of HCl for test portion during titration; M : molarity of HCl solution; W_m : weight, g, of sample; W_f : weight, g, of 20 ml filtrate; W_t : weight, g, of TCA plus sample solution; a : Factor that was obtained by the sum of the %, fat and %, total protein content.

Protein content, % = Nitrogen, % x 6.38 Eq 2.6.

2.2.5.4. Fat Content

Fat content of samples were determined by using Soxtherm automatic extraction system (Gerhardt GmbH & Co., Bonn, Germany). Hexane was used as solvent. Firstly the beakers were weighted; (M1) before extraction. Then, the weighed samples (M3) were completely involved in boiling solvent at 135˚C. The extractable matter was released from the sample. The matter was extracted by refluxing Hexane and was saved in the extraction beaker. Finally, fat contents of samples were calculated by using Eq. 2.7.

$$\text{Fat content, \% (wt/wt)} = \frac{(M2-M1)}{M3}\ x\ 100 \qquad \text{Eq 2.7}$$

2.2.5.5. Mineral Content

Mineral content of samples were determined by scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM-EDX) technique. (Quanta 250FEG; FEI, Oregon, USA). Atomic structure is unique so X-ray emission spectrums are different for each element. Therefore this technique allows to measure elemental composition of samples.

2.2.5.6. Water Binding Capacity

Water binding capacity was determined using the method reported by Yu et al. (2007). Three gram of sample was weighed into a 50 ml centrifuge tubes, which of weight was known. For each sample, 30 ml of distilled water was added, and blended with a vortex (Velp Scientifica, Usmate, MB, Italy) at 2400 rpm for 2 min. Then, samples were stood at room temperature for 30 min, and centrifuged at 2600 x g for 20 min. The supernatant was poured and centrifuge tubes containing precipitate were weighed. Water binding capacity was calculated according to Eq. 2.8

$$\text{WBC} = \frac{(W2-W1)}{W0} \qquad \text{Eq 2.8}$$

Where, WBC is water holding capacity (grams of water / gram of sample), W_1 is the weight of the tube and the dry sample (g), W_0 is the weight of the dry sample (g), and W_2 is the weight of the tube and precipitate (g). All the CGMP isolates and whey were analyzed.

2.2.5.7. Lactose Analysis

Lactose content of samples was measured with Perkin-Elmer Series 200 HPLC (USA) system with 87P column. Samples were diluted (1:29) with ultrapure water which was the used as mobile phase. Lactose standards were prepared at different concentrations (0.5 mg/ml – 1.0 mg/ml) with ultrapure water in order to graph the standard calibration curves. All the samples and standard solutions were syringe filtered through 0.45 μm filters. The flow rate 0.6 ml/min and the column temperature was maintained at 80°C.

2.2.6. Statistical Analysis

The data from caprine whey was analyzed by a one-way ANOVA (Minitab 16.1, Minitab Inc., Coventry, UK) and least significant difference test with significance at $p<0.05$ were used to compare the means.

CHAPTER 3

RESULTS and DISCUSSION

In this section isolation and characterization of CGMP from caprine whey are presented.

3.1. Isolation Methods and Protein Fractions of Casein glycomacropeptide

There are several methods described to isolate CGMP from whey such as high performance liquid chromatography (HPLC), ion exchange chromatography, hydrophobic interaction chromatography, gel chromatography and membrane separation techniques. Membrane techniques are reported to be the most particularly appropriate for food industry because of the comparatively easy scale up and low proceeding cost in comparison to chromatographic techniques (Tolkach and Kulozik, 2005).

3.1.1. Membrane Process

In Experiment1, a membrane technique described by Martin-Diana et al. (2002) was used in order to isolate CGMP from whey proteins. Chromatogram of whey is given in Figure 3.1. Retentate was collected as CGMP isolate. The chromatogram of Retentate is shown in Figure 3.2. As can be seen, the concentration of whey proteins was not effectively decreased, as was unexpected because these results were not in accordance with Martin-Diana et al. (2002), who reported that CGMP can be isolated with high purity (85.7% for caprine whey) after thermal denaturation and ultrafiltration. The peaks for α-lac appeared at retention time between 23.7 and 24.5 min, while the peaks corresponding to β-lg typically appeared between 25.3 and 26.06 min. β-lg concentration was decreased by 81,3% after heat treatment in Experiment 1.

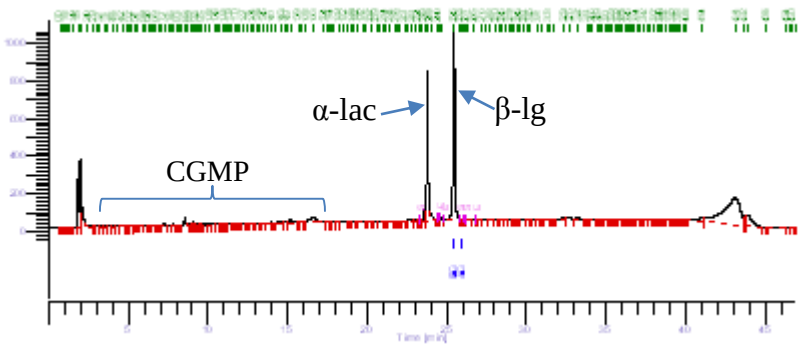


Figure.3.1. Chromatogram of C-Whey in Experiment 1

Whey proteins are heat-labile proteins. Their stability is decreased with the increasing temperature. In general, heat causes the denaturation of proteins, which occurs in two phases. First phase includes unfolding of the proteins which may be irreversible or reversible. First phase is followed by aggregation, which involves the interactions of proteins that are reversible or irreversible. Generally, aggregation follows an irreversible unfolding (Jovanovic et.al., 2005; de Wit and Klarenbeek, 1984; Ryan et.al., 2012).

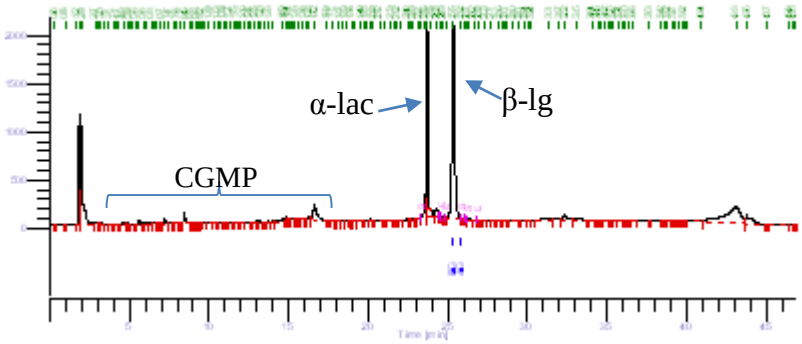


Figure 3.2. Chromatogram of Retentate in Experiment 1

β-lg is the most abundant protein in whey (Thoma et.al., 2006), and its thermal stability is higher than other whey proteins (Bonnaillie et.al., 2014). Therefore, the denaturation state of whey proteins may be determined by the structural change in β-lg.

Predominantly β-lg reveals as a dimmer which made of two subunits which is exactly identical in aqueous solution at room temperature and pH 5-7. It involves two disulphide bonds and one sulfhydryl group which is buried within β-lg. In case of denaturation by several agents (including heat), sulfhydryl group of β-lg is exposed and

become active and then formed sulfhydryl-disulphide interactions with itself or other proteins (Jovanovic et.al., 2005; Arriaga, 2011).

Numerous previous study describe the thermal denaturation of β-lg (Jovanovic et.al., 2005; Arriaga, 2011). Ordinarily, thermal denaturation of β-lg occurs in two phases, primary and secondary (Arriaga, 2011; Jovanovic et.al., 2005; Krichmeier et.al., 1985; Morr, 1985; Elfagm and Wheelock, 1978a; 1978b; Sawyer, 1968). In the first phase, four monomers arise by dissociation of dimmers and undergo interactions with sulfhydril groups and compose small aggregates (Arriaga, 2011; Jovanovic et.al., 2005). This composition occurs at temperature ≥70°C (Arriaga, 2011; Jovanovic et.al., 2005; Krichmeier et.al., 1985; Morr, 1985; Sawyer, 1968) and velocity of aggregation reaches the maximum value at a range of 80-85°C, (Jovanovic et.al., 2005; Donovan and Mullvihill, 1987; De Rham and Chanton, 1984;). In the second phase high molecular weight aggregates are composed by the interaction of small aggregates by the way of nonspecific interactions (Arriaga, 2011).

β-lg is prone to dissociation and association which is dependent of pH. β-lg formas a stable dimmer at pH 5-7. But, at pH ≤ 3.5 β-lg dissociates reversibly into monomers because of the high electrostatic repulsion (Boye et.al., 1996; Dalgalarrondo et.al., 1990). This means, at this pH range β-lg has a high net positive charge, so the proteins in the solution have many repulsive forces between them. These forces restrains the interaction of proteins, even though heat is applied (Martin-Diana et.al., 2002). Consequently, heat stability of β-lg is higher at pH ≤ 3.5 and maximal thermal stability of β-lg was identified at pH 3.0 (Boye et.al., 1996). On the other hand electrostatic repulsion can be eliminated. At isoelectric point (pI), the net charge of protein is zero. Thereby, protein-protein interactions can be occurred more easily at pI of proteins and the pH range of 3.2 to 3.9 is the range for CGMP isoelectric point (pI). Yılmaz-Gemili (2012) was also determined the minimum number of charges in order to reduce the electrostatic repulsion and inhibit aggregation at pH 3.8 to 4.6. Because of all these reasons and features which were mentioned above, pH value of Step 1 in Experiment 1 should be modified (Balestrieri, 1978).

The isoelectric points of α-lac and β-lg is between pH 4.8-5.3 (Arriaga, 2011). However, several studies were reported that, α-lac cannot precipitate at its pI because of its hydrophilic character (Swaisgood, 1986; Eigel, et al., 1984; Gordon and Kalan, 1974). Askonas (1954) gave the pI value of the goat β-lg in 5.9. Thus, it was selected for a pH value close to pI value of β-lg of Step 1.

Pintado and Malcata (1995) have been reported that, the most important effect for protein precipitation was temperature for caprine whey. Previous studies indicated that, β-lg and α-lac remains soluble at T≤ 85°C, though IgG and BSA disappear from solution. Complete precipitation of β-lg and α-lac occurs only on heating to over 90°C and β-lg and α-lac precipitate completely at 95°C (Ramos, 1978; Robinson, 1974). On the other hand the heat resistance of CGMP is higher than β-lg and it is not denatured at temperature above 90°C (Bonnaillie et.al., 2014; Jovanovic et.al., 2005). Starting from these information, heat treatment of Step 1 in the Experiment 2 was applied at 95°C.

In Experiment 2, the temperature, pH value, and processing time of thermal precipitation were increased. It was decided to apply thermal precipitation at 95°C for 120 min. The pH value of whey was adjusted to 5.2 with 1 M HCl before heat treatment. Centrifugation step was also modified to be more efficient. It was applied at 10,000 x g, at 4°C, for 20 min. Furthermore, diafiltration step was added in the isolation process in order to reduce the lactose content and to improve the purity of CGMP isolate. Retetante 2 was collected as CGMP isolate. The chromatograms, related to Experiment 2, were shown in Figure 3.3 and Figure 3.4. When the chromatograms have been examined, it can be seen that, the large part of whey proteins (99,92% of β-lg) except CGMP was precipitated , and CGMP was isolated with 44.23% purity in Retentate 1 (R1) and after diafiltration, CGMP was obtained with 88.55% purity and β-lg content was decreased by 99,96% in UF-CGMP.

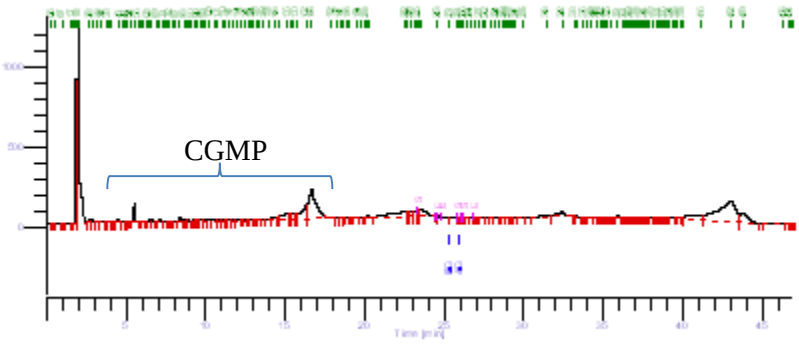


Figure 3.3. Chromatograms of Retentate1 (R1) in Experiment 2

Based on these results; thermal precipitation method of Experiment 2 was found effective to isolate CGMP with high purity (88.55%) and has been better than Experiment 1 which yielded to remove most of the whey proteins. However, physico-chemical

properties of other whey proteins were changed irreversibly. In other words they were denaturized. Whey proteins especially β-lg have very valuable functional and nutritional properties. Therefore, the unusable state of these proteins would be a great loss. On the other hand, β-lg is substantially allergic (Host and Halken, 1990; Bush and Hefle, 1996) and reduction the concentration of β-lg in the first instance would explore the usage of whey proteins in food technology. For these reasons, β-lg was attempted to be isolated from the whey before heat treatment; being denatured as seen in Table 3.1.

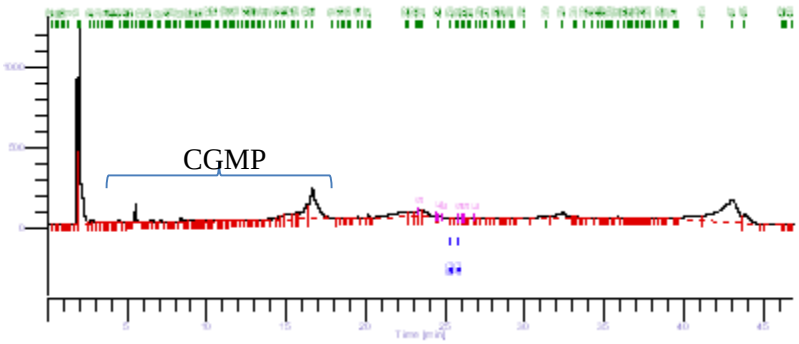


Figure 3.4. Chromatograms of UF-CGMP in Experiment 2

3.1.2. Chitosan Precipitation

In Experiment 3, β-lg was attempted to be isolated before thermal denaturation step. A method described by Casal et al. (2006) was used for selective isolation of undenatured β-lg from whey. The method is taken advantage of interactions between chitosan and β-lg. Chitosan is positively charged and operating pH value was selected according to the value of pH at which the β-lg is negatively charged.

First precipitation occurred at pH 4.5. This precipitation was done in an attempt to reduce turbidity. Lipids in the whey which is negatively charged was induced to turbidity and lipids should be removed not to inhibit chitosan- β-lg interactions. Clarified whey, obtained from first precipitation, was treated with chitosan at pH 6.2 where precipitation of β-lg was reached the maximum level and then supernatant was collected. Samples that were taken at intervals (15, 30, 60 min, 4, 24 h) were analyzed by RP-HPLC. β-lg contents are presented in Table 3.1. Chromatograms of Supernatant 3.2 and R-Whey are shown in Figure 3.5 and Appendix C, respectively.

Table 3.1. β-lg contents remained in Supernatant 3.2

	15 min	30 min	1 h	4 h	24 h
β-lg contents (%, wt/wt)	92.63	91.62	87.34	84.73	36.61

In the reference study, it was reported that, at least 10% of the rest of the β-lg was remained in the Supernatant 3.2. In Experiment 3, β-lg content was significantly decreased after 24 h, but 36.3% β-lg was still remained in Supernatant 3.2. This process was not as effective as referenced study.

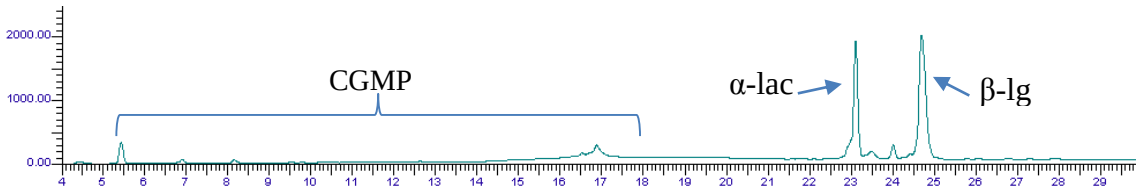


Figure 3.5. Chromatogram of Supernatant 3.2 in Experiment 3

Relationship between temperature, pH and concentration of salt is highly effective on aggregation behavior of β-lg (Arriaga, 2011; Mounsey, et.al., 2008; Jovanovic, et.al., 2005; Pintado and Malcata, 1995). Mounsey et al. (2008) found that, formation of insoluble β-lg-chitosan complex is inhibited by presence of salt at pH values >5.0. Whey, produced in the laboratory, was used for Experiment 3 and salt was not added during manufacturing. Thereby salt concentration did not have an effect on insoluble complexation between β-lg and chitosan in this experiment.

The operating temperatures for both precipitation processes were not given in the referenced method, so Experiments 3 were carried out at 25°C in this thesis study. Denaturation temperature of β-lg can be effectively changed by the pH of the supernatant to be heat treated. Thus the pH value of the supernatant containing β-lg becomes an important parameter. In addition, Mounsey et al. (2008) reported that protein insolubility level is reached to maximum (92%) at pH 6.0 as long as heat treatment was applied at 78°C for 10 min in the presence of chitosan. All these results and features, which were mentioned above, would be indicated that chitosan prevents the denaturation and promotes the aggregation of β-lg with heat treatment at pH values above 5.4. Therefore, operating temperature of chitosan treatment should be modified for significantly effective precipitation.

On the other hand, CGMP from bovine whey and CGMP from caprine whey were used in referenced study and in presented study, respectively. It was indicated that chemical compositions of bovine and caprine CGMP and thus their pI values are different (Robitaille et.al., 2012; Hernández-Ledesma et.al., 2011; Silva-Hernandez et.al., 2002). Thereby, repulsive forces of β-lg at studied pH and aggregation mechanism between caprine CGMP and chitosan might be different. Because of all these reasons, the method used might not have been as effective as the reference method.

3.1.3. SDS-PAGE Analysis

Soluble fractions of samples were analyzed to determine the distribution of whey proteins using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Figure 3.6, 3.7 and 3.8 show the protein proportions of the samples as approved with molecular mass marker, α-lac and β-lg standards. Whey solutions remarkably contained more β-lg than α-lac and very small amount of other whey proteins. After heat treatment process at 95°C at pH 5.2, there were appreciable difference in protein proportions (Figure 3.8) between whey and heat treated whey. Non-negligible amount of β-lg and α-lac precipitated during heat treatment in Experiment 2. On the other hand, Figure 3.7 shows that whey protein precipitation during heat treatment process at 80°C at pH 3.5 in Experiment 1 was not as effective as heat treatment process in Experiment 2.

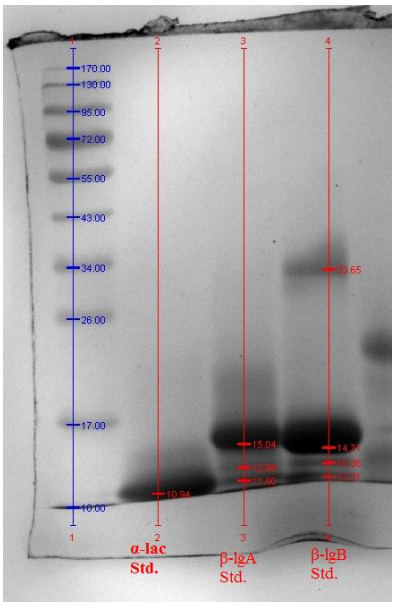


Figure 3.6. SDS-PAGE profiles of whey protein standards.

SDS-PAGE analysis is based on the estimation of related molecular mass to determine the amount of major proteins in the samples and the dispersion of proteins among sample fractions (Bonnaillie et.al., 2014; Coolbear et.al., 1996b). In this analysis, mini gel was used, thereby a marker that provides the opportunity to analyze the molecular mass from 10 to 170 kDa was run. According to Farias et al. (2010) the molecular mass of CGMP depends on genetic variant and average molecular mass of total CGMP distributes from 7.5 to 9.631 kDa. On the other hand Kawasaki et al. (1993b) reported that molecular mass of CGMP is pH dependent and it distributes between 20 and 50 kDa at pH 7 and 10 and 30 kDa at pH 3.5. Therefore, SDS-PAGE analysis can be used for the detection of whey proteins but CGMP could not be detectable on the SDS-PAGE gels, therefore samples were also characterized by RP-HPLC (Bonnaillie et.al. 2014).

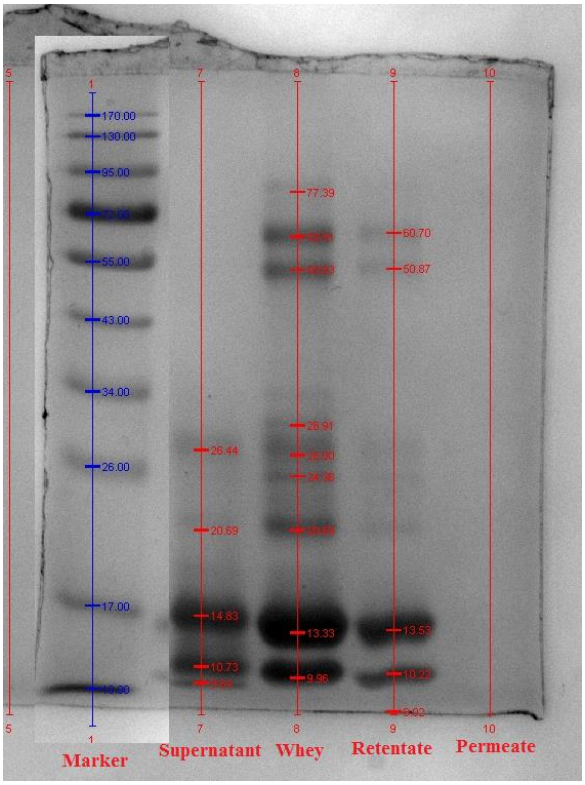


Figure 3.7. SDS-PAGE profile of Experiment 1

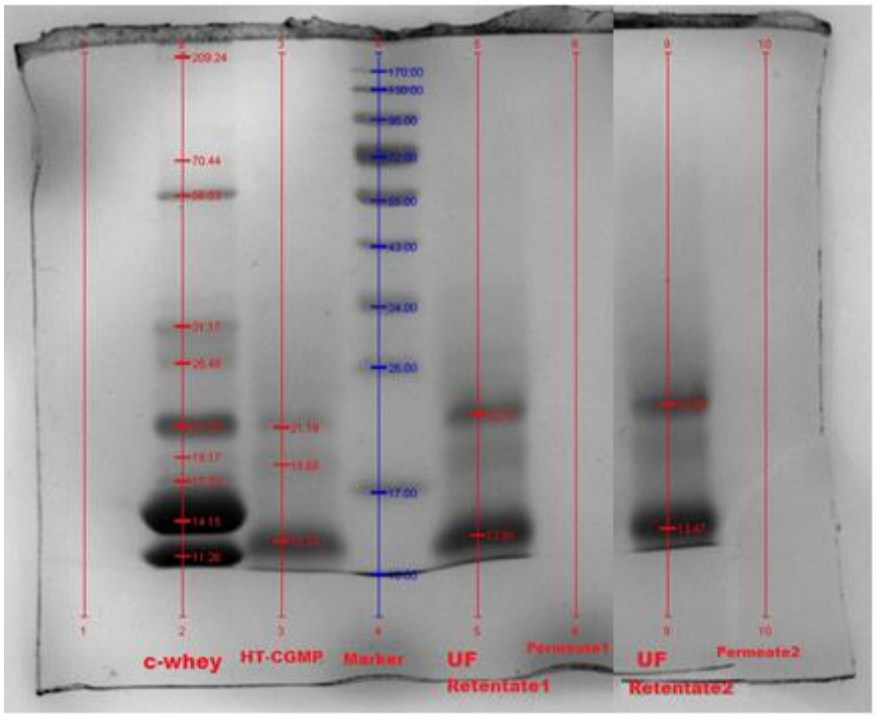


Figure 3.8. SDS-PAGE profile of Experiment 2

Since the molecular weight of β-lg was low (app. 8 kDa) it is difficult to visualize with Coomassie Blue stain in SDS-PAGE. Besides Trichloroacetic acid precipitation is recommended to clarifie other whey proteins in order to leave the CGMP in the solution phase (van Hooydonk and Olieman, 1982; Sharma et.al., 1993). In another study, it was estimated that CGMP is anionic and the affinity of SDS is insufficient to dissociate the dimeric CGMP (Silva-Hernandez et.al., 2002). Bonnaillie et al. (2014), Holland et al. (2010), Mikkelsen (2006) and Coolbear et al. (1996b) also conducted SDS-PAGE analysis to detect protein composition of whey and crude protein isolates. Bonnaillie et al. (2014) and Holland et al. (2010) did not determine CGMP content while Mikkelsen (2006) and Coolbear et al. (1996b) have shown CGMP on gels.

3.1.4. Purity Control of Casein glycomacropeptide

The CGMP chromatographic purity of the samples taken from different isolation steps is shown in Table3.2. Because CGMP standard could not obtained in commercially CGMP retention and resolution were published in the literature has been selected as a representative standard for the appropriate analogy. Detected by reference to the work done previously (Martin-Diana et.al., 2002). The obtained chromatograms of CGMP and

whey was comparable to chromatograms obtained in the studies reported by Bonnaillie et al. (2014) and by Martin-Diana et al. (2002).

Table 3.2. Characteristics of CGMP obtained from caprine whey in Experiment 2 and Experiment 3

Samples (%)	C-whey	Supernatant1	R1	UF-CGMP	R-whey	CP-CGMP
Purity	23,15	62.10	45.23	88.55	23,2	26.05
Glycosylated CGMP	8.42	12.22	6.31	4.15	7.26	12.76
Non-glycosylated CGMP	14.73	49.89	35.67	84.4	15.96	13.28

The degree of purity of heat treated (HT-CGMP) and ultrafiltrated CGMP (UF-CGMP) were slightly lower than CGMP obtained in other studies. Martin-Diana et al. (2002) obtained caprine CGMP with 85.7% purity. On the other hand, diafiltration process in Experiment 2 increased the purity up to 88.55% and 77.97% of CGMP was recovered. Li and Mine (2004) found the UF process as the most effective and they recovered 33.9% of CGMP. Bonnaillie et al. (2014) succeeded to isolate CGMP with 94% purity by using supercritical CO_2 processing and membrane filtration. Karakaya (2012) reported that, 57.56% CGMP was obtained. Among the reported values, CGMP has been isolated with high purity in this study.

3.1.4.1 Determination of Phe-Tyr Content by RP-HPLC

Phenylalanine and Tyrosine contents of lyophilized whey and CGMP isolate were determined by RP-HPLC. All samples were acid hydrolyzed before further analysis. Standard calibration curves of Phe and Tyr and chromatograms of standards and samples are given in Appendix B and Appendix D, respectively.

Contents of these amino acids in samples were determined by the calibration curves. CGMP isolates and lyophilized whey contain 0.858 mg Phe / g isolate and 1.69

mg Phe / g whey respectively. As can be inferred from results; whey contain more Phe than CGMP isolates (0.05>p).

Lim et al. (2007), used a commercial CGMP that contains 4 mg Phe / g CGMP, in product development for individuals with PKU. Doultani et al. (2003) and Etzel et al. (1999) have succeeded to isolate CGMP with Phe concentration less than 2 mg Phe / g CGMP. Karakaya (2012), obtained CGMP isolate that contained 3.305 mg Phe per g CGMP isolate. Martin-Diana et al. (2002) reported that Phe content of CGMP, that was isolated from goat's whey by heat treatment-UF, was 2.3%.

Phenylalanine tolerance can be varied considerably related to PKU (MacDonald, et.al., 2011) but it has been reported that Phe consumption should be less than 500 mg/day in a strict low-Phe diet (Bickel et.al., 1953; Wendel et.al., 1990). Thereby, ingredient that developed in this work was suitable for PKU diet.

3.2. Compositional Analysis

Table 3.3 shows overall composition of whey and CGMP isolates.

Table 3.3. Chemical composition (%, wt/wt) of caprine cheese whey and CGMP isolates.

	Ash	Dry Matter	Protein	NPN	Fat
C-Whey	9.3	93.25	0.92	1.35	2.28
UF-CGMP	9.87	93.09	0.35	1.38	0.13
R-Whey	8.43	93.87	1.57	1,23	6.18
CP-CGMP	13.13	93.8	0.08	1,24	0.054
HT-CGMP	9.65	93.84	0.97	1,23	0.73

3.2.1. Dry Matter Content

Dry matter content of HT-CGMP and R-whey were similar (p>0.05). This shows that heat treatment applied to the whey did not change the dry matter content. In addition, dry matter content of UF-CGMP was lower than C-whey (p<0.05). Namely UF process decreased the dry matter content. Chitosan precipitation also reduced the dry matter content of whey but it was not as effective as UF.

Dry matter content of liquid rennet caprine whey (R-whey) (7.1%) was also determined. It was similar to that reported by Sanmartín et al. (2012) and Moreno-Indias et al. (2009), lower than that observed by Pintado et al. (1999), and higher than that found by Casper et al. (1998) and Pintado et al. (2001)

3.2.2. Ash Content

Ash content of whey samples were increased after heat treatment and UF process ($p<0.05$). Chitosan precipitation also increased the ash content of CGMP isolate. Small increases in ash contents of CGMP isolate were observed during other processes applied. These values of ash contents after heat treatment and UF process were, 14.52% and 9.41% in the case of heat treatment and UF processes, respectively.

The ash content of liquid R-whey (0.65%) was similar to that found in sweet caprine whey but lower to that observed in acid caprine whey by Casper et.al., (1998). The mineral content of sweet whey is lower than acid whey through solubilisation of colloidal calcium phosphate of casein micelles that arises with acidification (Sanmartín et.al., 2012; Jelen, 2003; Pintando et.al., 2001).

C-whey had higher ash content than R-whey ($p<0.05$). The process differences such as curd grain size, temperature, and the speed and degree of acidification effects the ash content in caprine whey (Park, 1990). All these factors may have caused the differences in ash content between whey samples.

Ash content of CP-CGMP is significantly higher than other CGMP isolates. That was probably related to chitosan residues in supernatant.

3.2.3. Fat Content

Fat concentration of R-whey was much higher than fat concentration of C-whey. This might be due to the fact that C-whey was obtained from a local cheese-maker and standardization process was applied in the factory. So this difference in fat content between R-whey and C-whey was expected. Chitosan precipitation process decreased the fat content by 99.13%. This decrease was higher than that reported by Casal et.al. (2006).

3.2.4. Protein and NPN Content

The protein content of samples are given in Table 3.3. Whey protein content was calculated by subtracting non-protein nitrogen (NPN) content from total nitrogen content. NPN analysis performed by the TCA precipitation. Saito et al. (1991) reported that NPN content includes CGMP. So this value may be given evidence about CGMP content in whey and isolates. Heat treatment and UF process decreased the protein content but NPN contents of C-whey and UF-CGMP were similar ($p>0.5$). Martin-Diana et al (2002) determined the protein content 8.2%, and Saito et al. (1991) reported that isolates have 6.0% protein content. All these results significantly higher than protein contents in this study.

3.2.5. Water Binding Capacity

Texture and flavor of foods are influenced by interaction of water with proteins. Water binding capacity of samples were determined by the method that was described by Yu et al. (2007). Protein conformation, amino acid composition, surface polarity/hydrophobicity are intrinsic factors that affect water holding capacity of food proteins (Barbut, 1999). On the other hand, food processing techniques have effects on the protein hydrophobicity and conformation. Observation and data obtained during this analysis showed that CGMP isolates did not hold water on the contrary they dissolved in water.

3.2.6. Lactose Content

Lactose content of samples were determined by RP-HPLC analysis as described in material and method section. Lactose content of caprine whey was 87.07%. After ultrafiltration Retentate 1 obtained with 85.73% lactose content and diafiltration process was reduced the lactose content to 56.089% in Retentate 2. Martin-Diana et al. (2002) used C-whey for ultrafiltration with the lactose content from 65 to 73%. Lactose concentrations of Retentate 1 and Retentate 2 were 35-46% and 7.1-8.2% respectively. The initial lactose content of whey was very high. Diafiltration process decreased the lactose content but was not effective.

3.2.7. Mineral Content

Mineral contents of samples were presented in Table 3.4. Martin-Diana et al. (2002) reported P content of caprine whey as 1.3%. Ca content of caprine whey determined as 0.04 % (g/100 g protein) (Sanmartín et.al., 2012) which excessively lower than results obtained in this study. This value is significantly higher than values obtained in this study. The contents of other minerals in two whey samples were varied. Rate of acid development and curd grain in the production procedure of cheese have been indicated as being influencing factor for the remaining of minerals in cheese and in whey (Park, 1990). SEM-EDX images of samples are presented in Appendix E.

Table 3.4. Mineral composition of samples

	P	**Ca**	**Mg**	**K**	**Na**	**Cl**
C-Whey	0.29	0.50	0.09	1.07	0.23	1.40
UF-CGMP	0.30	0.70	0.10	1.42	0.33	1.72
R-Whey	0.34	0.30	0.10	1.50	0.20	0.90
HT-CGMP	0.60	0.34	0.13	1.66	0.26	1.31

CHAPTER 4

CONCLUSION

CGMP was isolated from caprine whey by using two different methods. Firstly, and Ultrafiltration (UF) process was used for the isolation of CGMP. Retentate was collected as CGMP isolate. In Experiment 1, β-lg content of whey was decreased by 81.3% after heat treatment. This loses are quite low. It has been decided to use a severe pretreatment before UF process. Therefore in Experiment 2, heat treatment was occurred at 95˚C, at pH 5.2 for 2 h. β-lg content was decreased by 99.92% after heat treatment in Experiment 2. In Retentate 1, CGMP was collected with 38.2% purity. In order to increase the purity of isolate, UF was assisted with diafiltration. CGMP was purified with 88.5% purity after diafiltration and this level is comparable with the current literature. In addition β-lg content was decreased by 99.96% after diafiltration step. Therefore, a diafiltration step is a need to obtain a successful separation with UF process.

Secondly, a chitosan precipitation was carried out to recover undenatured β-lg. During isolation process of CGMP with UF, heat treatment denatured and precipitated most of whey proteins mainly β-lg which has valuable functional and biological activities. For this reason, undenatured β-lg precipitated with chitosan before heat treatment prior to UF process in Experiment 3. After 24 h shaking, 36.61% β-lg was remain in whey.

The protein fractions of whey and isolates were analyzed and characterized by SDS-PAGE and RP-HPLC analysis. Quantitative data was obtained from RP-HPLC analysis which is gives remarkable results when compared to SDS-PAGE analysis. The protein peaks were achieved with a good resolution in RP-HPLC analysis. No interference were observed.

As a result of isolations from the entire processes, it was decided that Experiment 2 is the most efficient method for CGMP isolation.

Phe-Tyr content of whey and CGMP isolate obtained from the most efficient isolation process was determined by RP-HPLC. Phe content of whey decreased by ....%.

Compositional characteristics of isolates and whey were determined with different methods. Two different whey were used during isolations. Whey obtained from a cheese-maker was used in the ultrafiltration process. Chitosan precipitations were also carried out in whey, solutions that was produced in the laboratory from fresh goat's milk. Both

two whey were having compositional differences, since, milk is treated by different processes in the factory. Therefore compositional differences between both whey samples are expected.

Ash contents of UF-CGMP and HT-CGMP is higher than C-whey and R-whey, respectively. However, an increase in the ash content of UF-CGMP was less than the increase in the ash content of HT-CGMP. This shows that, heat treatment increased the ash content but ultrafiltration process decreased it. Dry matter content of R-whey and HT-CGMP is similar ($p>0.5$), but dry matter content of UF-CGMP was lower than C-whey, so ultrafiltration process also decreased the dry matter content and heat treatment was not affected on it. Heat treatment and centrifugation step afterwards were decreased the protein content. However after the ultrafiltration process, protein content was reduced more. NPN contents that include CGMP were similar with each other in two experiments ($p>0.5$). It may be concluded following, heat treatment and ultrafiltration processes were not affect the CGMP content significantly. Fat content of both CGMP isolates decreased after heat treatment; ultrafiltration process removed the more fat from CGMP isolates.

The overall aim of this thesis is development of a novel ingredient based on CGMP and different isolation methods have been presented. The remarkable differences of this work from previously reported literature can be given as follows:

- CGMP was isolated from caprine whey instead of bovine whey. In this way, allergic risk of the ingredient was reduced.
- Food products for PKU are synthetic mixtures and most of them include high amount of food additives. The products usually produced as drugs rather than nutraceuticals.
- In this study, the main target is developing of a new food ingredient/product which is suitable for PKU patients, with a desirable taste and without any chemical food additives.
- Whey is a byproduct of cheese manufacturing. It is a pollutant because of its high biological oxygen demand. 100 kg milk releases 80-90 kg whey. This byproduct should be annihilated or collected as food ingredients. Otherwise drainage it through sewage damages to environment worryingly.

There are an estimated 15.000 individuals with treated PKU in the US and 50.000 worldwide, Turkey has the highest prevalence in PKU. In Europe and US special foods

produce for these individuals but in Turkey it is very difficult to reach this special foods. So this MSc thesis work will hopefully make contributions and knowledge to present this kind of functional foods for food industry.

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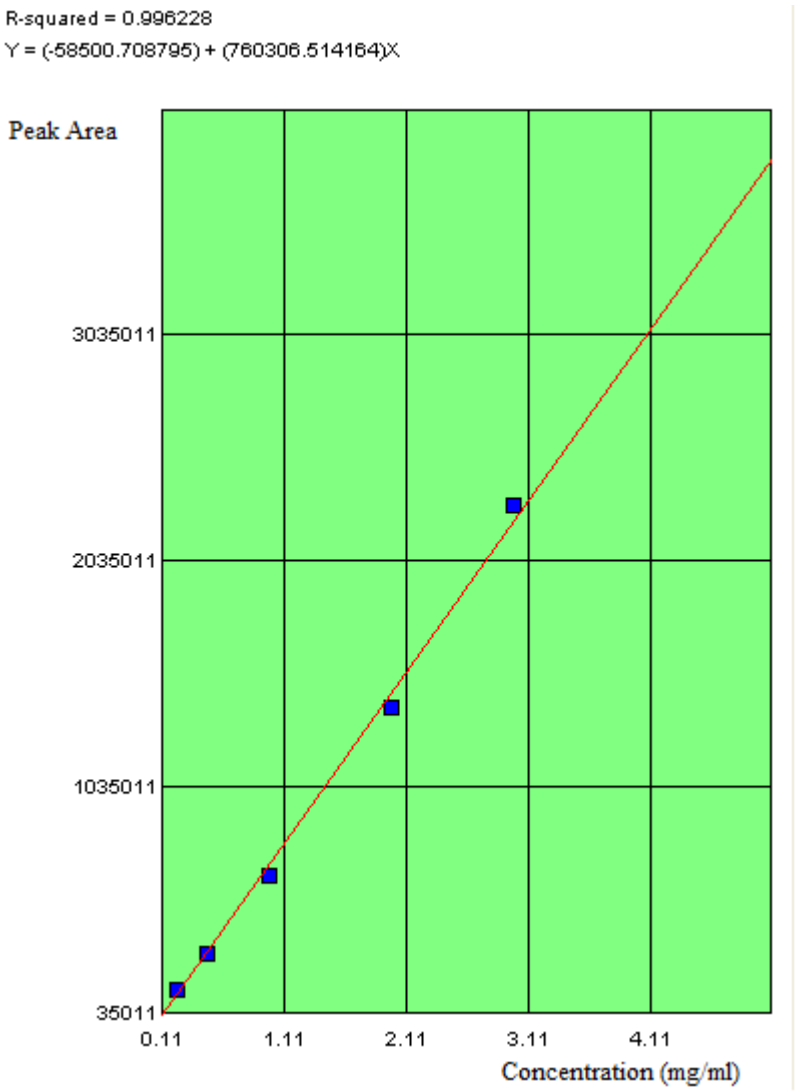
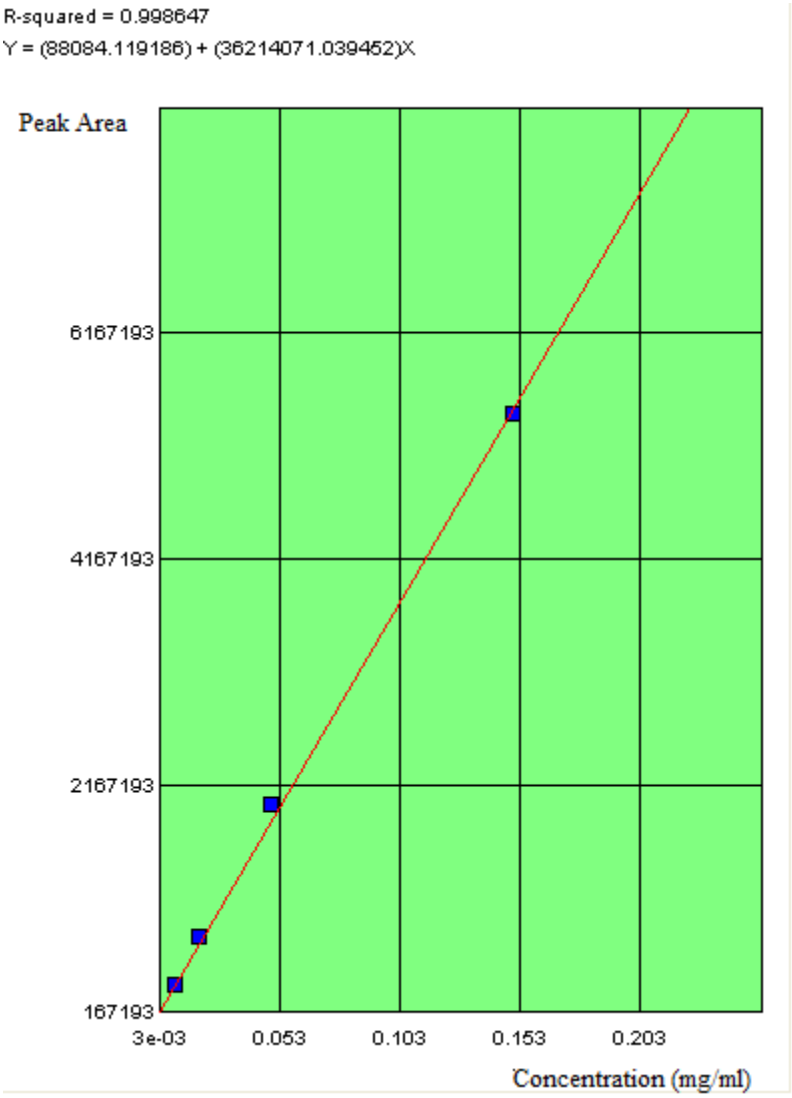
APPENDIX A

CALIBRATION CURVES

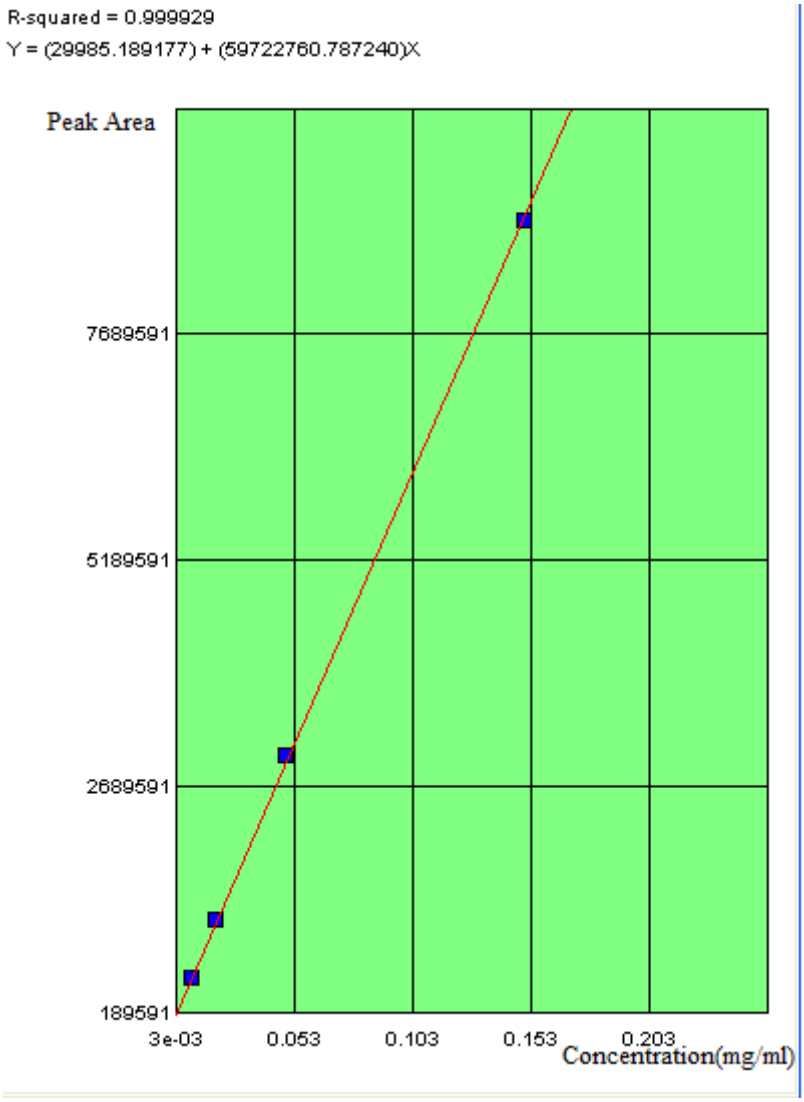
A1. Calibration Curve of standard β-lactoglobulin



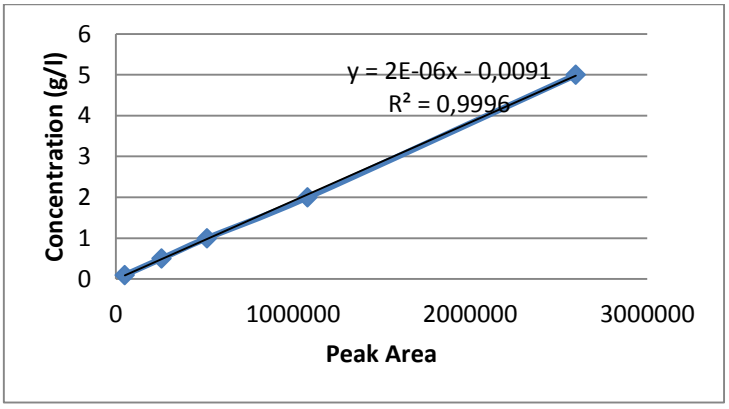
A2. Calibration Curve of Standard Phenylalanine



A3. Calibration Curve of Standard Tyrosine



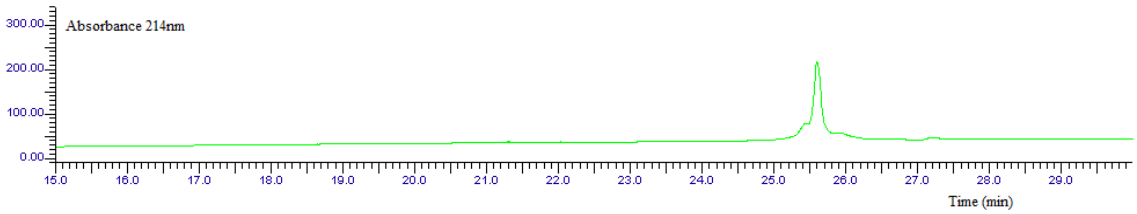
A3. Calibration Curve of Standard Lactose



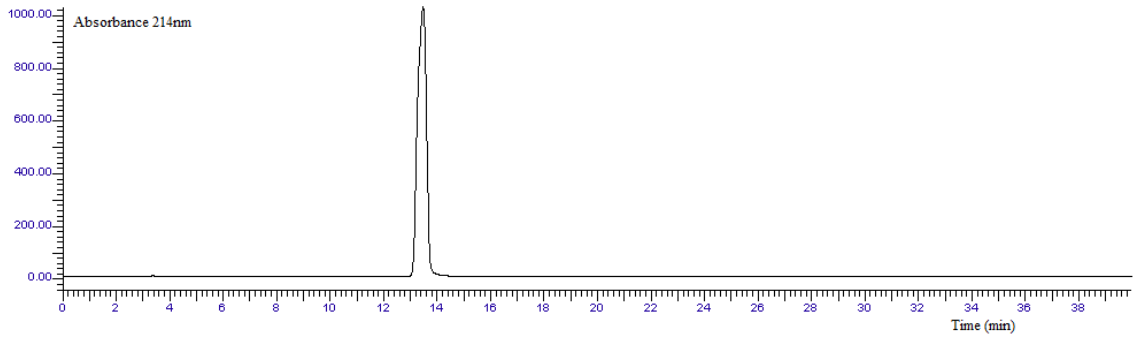
APPENDIX B

CHROMATOGRAMS OF STANDARDS

B1. HPLC Chromatogram of Standard β-lactoglobulin



B2. HPLC Chromatogram of Standard Phenylalanine

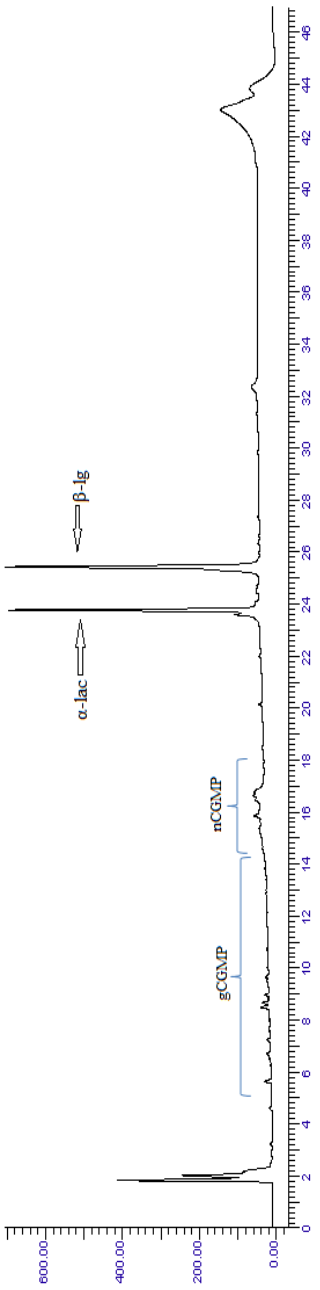


B3. HPLC Chromatogram of Standard Tyrosine

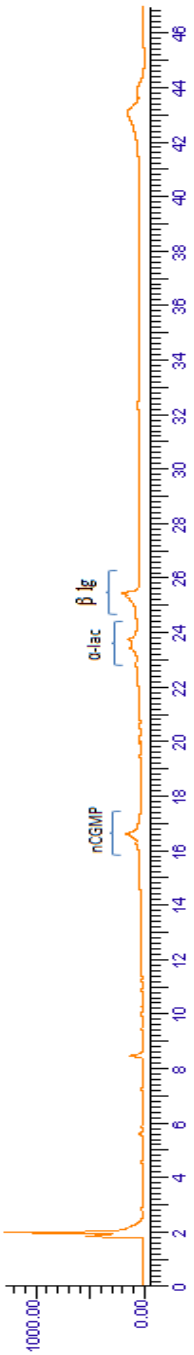
APPENDIX C

CHROMATOGRAMS OF SAMPELS

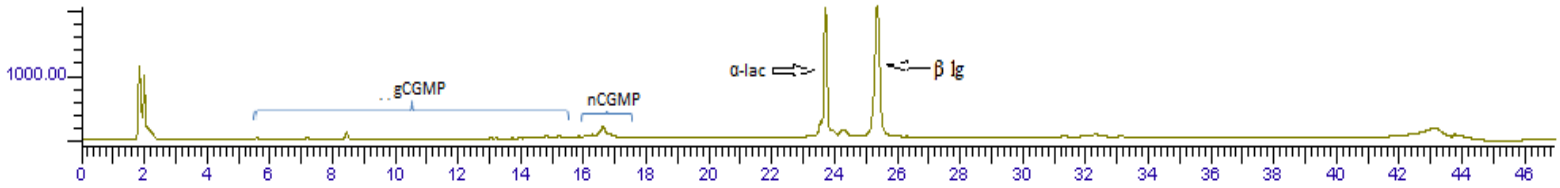
C.1. **HPLC Chromatogram of C-Whey**



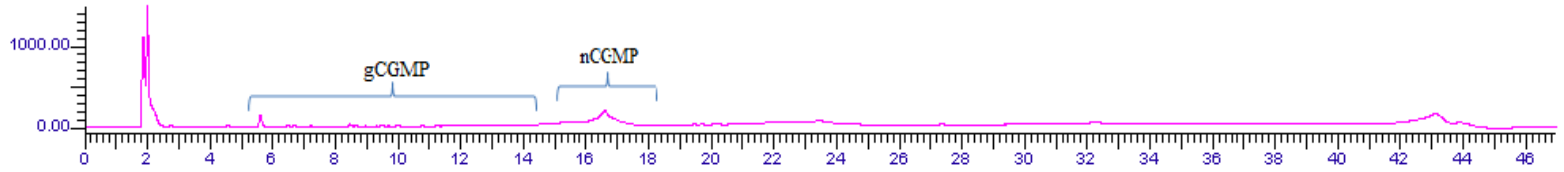
C2. HPLC Chromatogram of Supernatant in Experiment 1



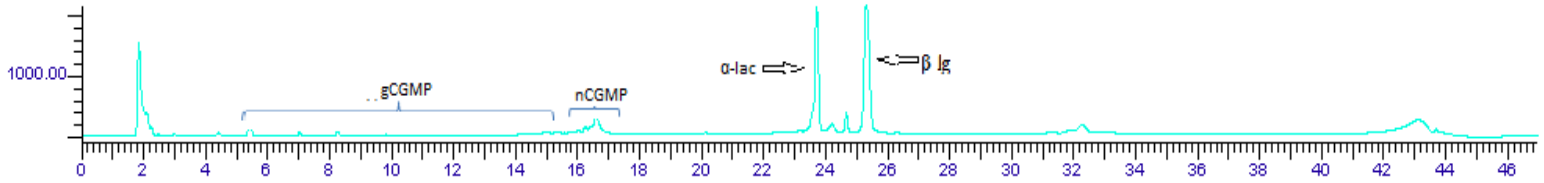
C3. HPLC Chromatogram of Retentate in Experiment 1



C4. HPLC Chromatogram of Supernatant 1 in Experiment 2



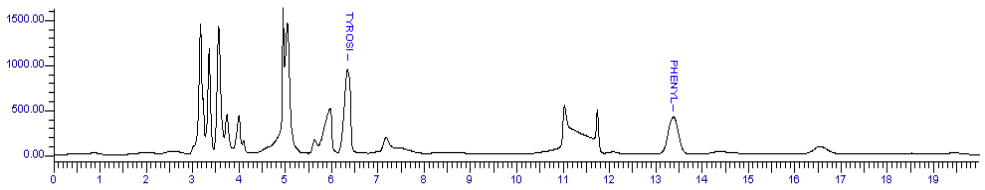
C5. HPLC Chromatogram of R-Whey



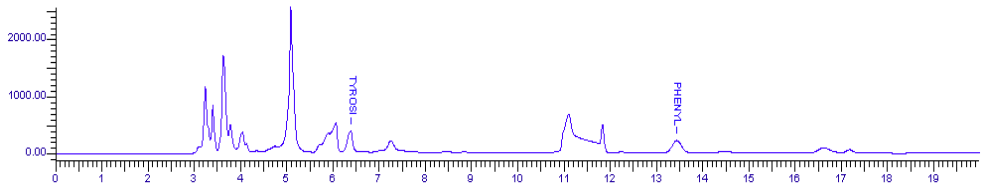
APPENDIX D

CHROMATOGRAMS of PHE-TYR

D1. HPLC Chromatogram of C-Whey



D2. HPLC Chromatogram of UF-CGMP

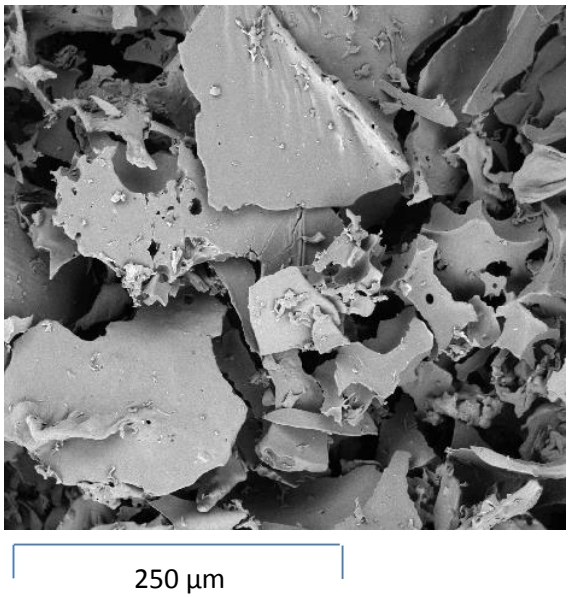


APPENDIX E

SEM-EDX IMAGES

E1. SEM-EDX Images of C-Whey

E2. SEM-EDX Images of UF-CGMP



E3. SEM-EDX Images of CP-CGMP