Glycomacropeptide Extraction

“Securing Glycomacropeptide and Casein Curd using GMP manufacture from skim milk”

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INTRODUCTION
Milk

- **Major component** of milk consist of 86.6% water, 5% lactose, 4.6% fat, 3.6% protein, and 0.7% ash.

- **Minor components** are enzymes, vitamins, and minerals (Swaisgood, 2007).

- Milk proteins are classified as either casein proteins or whey proteins (Dauphas, 2008).
Casein consists of 80% of the proteins in milk

Four main types: $\alpha_{s1}$-, $\alpha_{s2}$-, $\beta$- and k-caseins.

Stability of the casein micelles attributed to negative charge & steric repulsion by the micropeptide region of k-casein flexibility.

Ca-induced interaction between protein molecules, electrostatic, hydrophobic & hydrogen bonding are responsible for the micelle integrity (Lucey, 2004).
Rennet

General Application

- Rennet is a natural complex of enzymes in any mammalian stomach to digest mother’s milk.
- Proteolytic enzyme coagulate milk causing separation of curds & whey.
- The active enzyme is called chymosin or rennin.
Rennet

History

- Plant coagulation was used for fermenting cheese since agent times in Portugal.
- These plants can be found in southern European countries used for cheese making but some do not consider them of high quality.
- Calf rennet (rennin) was widely used until the 1970s (Esteves 2001).
- A growing demand for alternative source of coagulatents because of the reduced supply of calf rennet.
- Vegetarians have called for vegetarian alternative.
The preparation of the lamb rennet (Irigoyen, 2000) is collected and dried in a ventilated place protected from light.

Once dried, the fat is removed from the external surface and cut into cubes. It is then mixed with salt and kept in darkness at 4°C.

It is dissolved in water and filtered.

It is then preserved in cold, dry and dark until used.
Chymosin (Rennin)

- Chymosin is added in the manufacturing of cheese.
- Chymosin is a proteolytic enzyme synthesized by chief cells in the stomach.
- It is secreted as an inactive proenzyme called prochymosin.
- It is most active in acidic environments.
- It causes the coagulation of cheese & hydrolyses some k-CN resulting in diffusing away of the micelles.
Chymosin Coagulation

- Leads to a decrease in the zeta potential by \( \sim 5-7 \text{ mV} \) (~50%).
- Reduces the electrostatic repulsion between rennet-altered micelles.
- Removal k-casein results in decrease in the hydrodynamic diameter by \( \sim 5\text{nm} \), & loss of stabilization, coagulation & curd formation.
- Chymosin cleaves k-casein of the casein micelle at 105-106 Phe-Met bond resulting in coagulation (Lucey, 2004).

- Vegetable & microbial chymosin are reported to more clotting than animal enzymes (Irigoyen, 2000).
Casein Curd

- Casein curd obtained from the coagulation of milk
- Facilitated with the addition of chymosin.
- Milk sour naturally will also produce casein curds.
- Casein curd products: cottage cheese, quark and paneer, yoghurt, sour cream and cultured buttermilk.
- Casein cheese curd: an important step in cheese making.
- Casein cheese solids: separated from whey in milk processing.
- Different treatments yield different products.
- Finished product aged to create mature rich cheese.
- Cheese range from “curds and whey” for soft cheeses to hard cheeses (Wisegeek, 2008).
Whey

- Whey protein represents 18-20% of total milk nitrogen content (Jovanovic, 2005).
- β-lactoglobulin is major milk serum protein.
- α-lactalbumin represents about 20% of the total serum protein.
- Whey contains immune globulins & serum albumins enzymes.
- β-lactoglobulin is rich in lysine, leucine, glutamic and aspartic acid.
- α-lactalbumin has a high binding capacity for calcium and protects against thermal denaturation (Beaulieu, 2007).
- Gelling properties of whey proteins show evidence that structure, hardness & stringiness are a potential to develop of new products (Span, 2008).
Whey- Uses

- Functional foods & nutraceutical have become of increasing interest (Beaulieu, 2007).
- An interest in non-fat yogurt have been demonstrated (Sahan, 2008).
- Whey protein have multiple functions such as foaming, gelatin, and emulsification and have water-binding and high solubility (Beaulieu, 2007).
- The gelling has been used to impart creaminess & superior texture to soups & sauces & used in salad dressing & mayonnaise type products (Jonson, 20002).
- Potential new products: Flavored cheese whey that could provide new health benefits and attract new consumers.
Glycomacropeptide (GMP)

- Glycomacropeptide formed during the enzymatic coagulation of milk using chymosin.
- It releases to milk serum due to the hydrolysis of k-casein peptide catalyzed by rennin in cheese.
- Structure, composition, biological activities, functional and technical are parts of GMP purity evaluation.
- 64 amino acids residue glycomacropeptide with various biological activities to be found in sweet whey (Tullio, 2007).
Benefits of GMP

- Products containing GMP cited as potential to helping with diabetes, obesity, and hypercholesterolemia (Etzel, 2004).
- GMP may inhibit intestinal infection/intoxication (Bruck, 2006).
- May inhibit cariogenic, plaque-formation bacteria that cause tooth enamel demineralization and subsequent enamel remineralization (Aimutis, 2004).
- GMP may inhibit activation of immune cells and could potentially serve as an anti-inflammatory effect on inflammatory bowel disease (Daddaoua, 2005).
- Further research & clinical trials must be done document any of these potential health benefits.
Justification

- Products made from casein curd: cheeses & yogurt cheeses.
- Steady increase in per capita production & consumption of natural cheese since 1980 (International Dairy Foods Assoc, 2007).
- 3.2% increase in sales & consumption of cheese from 2005 to 2006.
- Consumption increase of 6.7% yogurt from 2005 to 2006 (International Dairy Foods Assoc, 2007).
- Curds can be used for new product lines.
- Type & texture of casein curd influences the type & quality of the product.
- Soft curds used: drinkable & fermentable drinkable products.
- Firm curds used: chewable & fermentable chewable products.
- Changing curd texture: financial implications dictated by type of product to be manufactured & contribution to product yield.
- Chymosin coagulation time may influence yield of GMP.
Hypothesis:

Chymosin coagulation time during GMP manufacture will influence curd attributes and GMP yield.
Objectives

- To study influence of chymosin coagulation time on physical characteristics of curd.

- To elucidate effect of chymosin coagulation time on the chemical attributes of curd.

- To determine impact of chymosin coagulation on microbiological characteristics of curd.

- To study the influence of chymosin coagulation during GMP manufacture on the yield & purity of GMP.
MATERIALS AND METHODS
Experimental design – Completely randomized design

- Casein Curd and GMP manufacture will be conducted on the same day but the treatments will be spread over various days.
- Treatments – chymosin coagulation times of 30, 90 and 150 minutes.
- Control – chymosin coagulation time typically used in cheese manufacture, 30 min.
- All three treatments will be randomly assigned to the experimental unit (milk lot).
- All physical, chemical and microbiological analyses will be conducted on fresh (day 1) casein curd.
- Replications – 3 replications will be conducted (casein curd and GMP manufacture will be repeated 3 times)
Chymosin coagulation, curd and GMP manufacture

- To 1 gal. of fat free milk add food grade 6N HCl until it reaches between pH 4.5-4.6.
- Casein allowed to precipitate & cook slightly until it reaches 50°C.
- Casein separated & drained completely from whey by filtration using several layers of cheese cloth.
- Casein will be washed three times with 1N HCl.
- Casein will be dispersed in a sodium citrate & calcium chloride solution.
- Casein will be mixed well & NaOH added until a pH 6.8 is reached.
- Homogenized.
- Casein will be then allowed to equilibrate at 32-37°C chymosin was added followed by gentle stirring.
- Left undisturbed for 30 / 90 / 150 min.
- Coagulum was obtained.
- Curd and GMP will be separated with several layers of cheese cloth.
- Curd and GMP will be set aside for further analyses.
Casein curd: Physical Analyses:

1. Apparent Viscosity
   - Viscometer will be used to measure the apparent viscosity of the curd.
   - Brookfield Wingather software will be used to collect data.
   - 30 readings will be taken per treatment per replication.
   - Average will be recorded in Excel program.
2. Color Measurements

- The L*, a*, b*, C*, & h values will be recorded using the Hunter Lab colorimeter.
- Color recording conditions will be D 65 and 10° observer.
- Universal software version 4.10 will be used.
- Average of five readings will be recorded per treatment per replication.
Chemical analyses

1. **pH**

- The pH electrode will be calibrated using pH buffers 7.00 and 4.00.

- pH will be recorded at room temperature (22°C).
2. **Titratable Acidity**

- A sample of 9 gram will be measured into a 100 mL beaker.
- Twice the amount of LG water as the sample will be added.
- The sample will be rinsed in a beaker and mixed gently thoroughly.
- 0.5 mL of phenolphthalein indicator will be added & titrated with 0.1 N sodium hydroxide to the first permanent color change to pink.
3. Moisture:

- Moisture of casein curd will be measured by placing a sample into a container.
- Sample will be placed in a steam bath for about 20 minutes and later in an air oven at 105°C until constant weight.
- The sample will then be cooled to room temperature (22°C) in a desiccator and weighed.
- Moisture % will be calculated using the following formula. 
  \[(\text{Sample before drying}) - (\text{sample after drying})/ (\text{sample weight before drying})*100.\]
Microbiological analyses

1. Total Plate Count

- Standard plate count will be performed using Plate Count Agar.
- Serial dilution of the samples will be made using 0.1% w/v phosphate sterile water.
- Plated, on Plate Count Agar, in duplicate.
- Petri dishes will be incubated at 32°C for 48 hours.
- After the incubation period, the colonies will be counted, with the aid of colony counter (Marshall, 1993).
2. **Coliform**

- Coliform Petrifilms will be used.
- Samples will be diluted in $10^{-1}$ dilution.
- The petrifilms will be inoculated with 1 mL of sample.
- Petrifilms will be incubated at 32°C for 24 hours.
- Red colonies with gas will be counted (Davidson, 2004).
3. Yeast and Mold:

- Yeast & mold count will be conducted using the yeast and mold petrifilms.

- The petrifilms will be incubated at 25°C for 5 days (Frank, 2004).
GMP Yield

- Amount of liquid GMP obtained will be measured.
- Trinitrobenzenesulfonic Acid (TNBS) method will be used in analyzing a sample of GMP.
- 1.0 ml of the sample analyzed (at a concentration of 0.01-0.87µ mol/ml).
- 1.0 ml of 4% sodium bicarbonate and 1.0 ml of 0.1% TNBS solution will be mixed.
- Sample will be kept in the dark at 40°C for 2 hours and will be measured at 340 nm after acidification with a defined volume of N HCl (Satake, 1960 and Mokrasch, 1967).
- 3 readings of each sample will be collected using the above method and place in a 96 well plate. This will be read in the microplate reader for analyses and read at 340 nm.
Following peptide concentration determination by the TNBS method, the purity of GMP will be evaluated by gel electrophoresis as follows.

- GMP was dissolved in water. Sample buffer was added to an aliquot of the GMP solution.
- The mixture was boiled in a water bath for 5 minutes, cooled, centrifuged and loaded on a gel for electrophoretic separation.
- Electrophoresis separation will be performed according to the manufacturer’s instructions.
- The gel will be stained with Coomassie Blue, destainer, and intensity analyzed for the presence of GMP and/or other peptide or protein impurity.
Data was be statistically analyzed (One-Way Analysis of Variance, standard deviation, difference in mean, and Multi Analysis of Variance (Bonferroni Test) using the SPSS statistical programs.

Significant differences was determined at $p < 0.05$. 


