5-17-2000

1999 Annual Report

Various Authors

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- Understand the role of proteolysis on functional properties of Mozzarella cheese
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PI: Donald McMahon and Joseph Irudayaraj  

Whitening of skim milk using a continuous-flow, immobilized flow, immobilized enzyme reactor  
PI: Marie Walsh  

Inhibition of lipid oxidation in precooked meats by dairy calcium and phosphoproteins  
PI: Daren Cornforth  

Developing an affinity purification procedure to purify prosaposin from whey  
PI: Marie Walsh  

Developing an extruded whey protein meat extender suitable for use in coarse-ground meat products  
PI: Marie Walsh  

Appetite suppressing properties of a peptide from milk  
PI: Deborah Gustafson  

Improvement of low-fat Cheddar cheese through identification and characterization of microbial enzymes responsible for conversion of aromatic amino acids into off flavor compounds in cheese  
PI: Jeff Broadbent  

Growth of non-starter lactic acid bacteria in reduced fat Cheddar cheese, Utah State University part  
PI: Jeff Broadbent  

Improvement of low-fat Cheddar cheese through identification and characterization of microbial enzymes responsible for the production or degradation of bitter peptides in cheese, Utah State University portion  
PI: Jeff Broadbent  

Microbial catabolism of methionine to improve Cheddar cheese flavor - a comparative study of the relative contribution by starter cultures and flavor adjunct bacteria  
PI: Bart Weimer
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The Western Dairy Center is a consortium of researchers devoted to improving the dairy industry in the United States by conducting research in all areas of dairy foods. The Center includes researchers from Utah State University, University of Idaho, Oregon State University, Brigham Young University, Washington State University and Weber State University. This report covers research activities from January 1, 1999 through December 31, 1999.

The Center Annual Meeting was held on May 20-21, 1999, at the Oregon State University Marine Science Center in Newport Oregon. A large group representing both dairy producers, processors and researchers attended and provided significant input onto the future direction of the Center.

The Center conducted the 15th Annual Cheese Making Short Course on Feb. 9-11, 1999, at Utah State University.

The Center of Excellence for Dairy Technology Commercialization, which is funded by the Utah State Office of Economic Development, continued to work toward commercialization products and technologies developed by researchers at the Western Center. Activities in 1999 concentrated on commercializing the exopolysaccharide cultures, the flavor injected cheese products, and the textured whey protein technology.

The Center for Microbial Physiology and Rapid Detection, directed by Bart Weimer, continued their activities in 1999. The center is developing methods to detect pathogens in dairy products, other food products, water and air.

In 1999, fifteen research projects were funded by DMI. Six research projects were funded by Center funds. Project progress reports of all research projects active in 1998 are included in this report.
Pursuant to the Western Dairy Center proposal and contract with the National Dairy Promotion and Research Board, the voting members of the Operational Advisory Committee are:

Eric Bastian  
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WESTERN DAIRY CENTER

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Carl Brothersen
Dept of Nutrition & Food Sciences
Utah State University
Logan, UT 84322-8700
Western Dairy Center  
Budget Report  
1998

Dairy Management Inc.  

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TOTAL Regional/Industry Support  

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<td>Total Committed Funds for 1999</td>
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1999 Balance  

$129,428.98
Financial Summary of Approved Projects for 1999

Projects Funded by DMI

Improvement of Mozzarella cheese functionality by understanding exopolysaccharide production in thermophilic starter cultures – Jeff Broadbent ................................................................. $41,190

Growth of non-starter lactic acid bacteria in reduced fat Cheddar cheese, Utah State University part – Jeff Broadbent ................................................................. $44,893

Improvement of low-fat Cheddar cheese through identification and characterization of microbial enzymes responsible for the production or degradation of bitter peptides in cheese, Utah State University portion – Jeff Broadbent – Charlotte Brennand ................................................................. $47,707

Improvement of low-fat Cheddar cheese through identification and characterization of microbial enzymes responsible for conversion of aromatic amino acids into off flavor compounds in cheese, Utah State University portion – Jeff Broadbent – Bart Weimer ................................................................. $52,983

Application of a metabolic control switch to improve and control starter proteolysis in Mozzarella cheese – Jeff Broadbent ................................................................. $38,519

The use of bacteriophage-receptor genes of Lactococcus lactis to develop bacteriophage-resistance in Cheddar cheese starter strains – Bruce Geller ................................................................. $103,985

Process technology to improve the flavor of heated milk – Joseph Irudayaraj – Don McMahon ................................................................. $48,403

Characterization of proteolytic enzymes from thermophilic lactic acid bacteria and their influence on Mozzarella cheese functional properties – Donald McMahon ................................................................. $56,182

Understand the role of proteolysis on functional properties of Mozzarella cheese – Donald McMahon ................................................................. $64,335

Developing an affinity purification procedures to purify prosaposin from whey – Marie Walsh ................................................................. $16,300

Developing an extruded whey protein meat extender suitable for use in coarse-ground meat products – Marie Walsh ................................................................. $28,000

Identification and characterization of components of the proteolytic enzyme system of Lactobacillus helveticus which effect bioactive peptide accumulation, Utah State University part – Bart Weimer ................................................................. $31,800
Financial Summary of Approved Projects for 1999 continued

Microbial catabolism of methionine to improve Cheddar cheese flavor – a comparative study of the relative contribution by starter and adjunct bacteria – Bart Weimer .......................................................... $73,470

Conversion of amino acids to short and branched-chain fatty acids by starter and adjunct bacteria – Bart Weimer .......................................................... $17,600

Controlling Chemical Composition and Functionality of Cheese – Conly Hansen .......................................................... $26,180

Projects Funded by WDC

Evaluate the effect of concentrated buttermilk fractions on iron transport and absorption – Deloy Hendricks .......................................................... $2,700

Effect of butter and margarine intake on human milk CLA and fat concentrations in lactating women – Mark McGuire .......................................................... $25,000

Determination of the oxidation/reduction potential of cheese – Bart Weimer .......................................................... $24,999

The proteome of lactic acid bacteria – Bart Weimer .......................................................... $10,000

Debittering with brevi protease – Bart Weimer .......................................................... $24,500

Effect of size, hydrophobicity and temperature on the diffusion of molecules within the Cheddar cheese matrix – Carl Brothersen .......................................................... $9,727
Western Dairy Center
Project Report
Reporting Period January 1, 1998 — December 31, 1999

Principal Investigators: Donald J. McMahon, Utah State University
Co-Investigators: Jeffery Broadbent
                    Craig Oberg

Project Title: Characterization of proteolytic enzymes from
thermophilic lactic acid bacteria and their influence
on Mozzarella cheese functional properties

Institution’s Project #: 98092

Project Completion Date: June 30, 2000

National Research Plan (1997): Priority: Goal: Tactic:
Understand role of moisture and proteolysis on physical and functional
properties of cheese.

Modifications to Project/Budget: None

Project Objectives: (Include any revisions to objectives)
Overall Objective: Understand the influence of microbial proteolytic systems
which have variable effects on the rheological properties of Mozzarella
cheese.

Objective 1: Characterize proteolytic enzymes in thermophilic Mozzarella
cheese starter cultures.

Objective 2: Investigate the influence of different distinct proteolytic enzyme
systems on Mozzarella cheese functionality.

Project Summary: (Suitable for inclusion in Center documents released to the
public)
The purpose of this project is to control the functionality of Mozzarella cheese
by understanding the role of starter culture proteolysis in the development of
melting properties. This project will investigate (1) the diversity that exists in
the proteolytic systems of important thermophilic starter cultures, and (2)
how these differences may influence Mozzarella cheese functionality. Strains
of lactobacill and Streptococcus thermophilus will be screened for proteinase
activity; specificity toward $\alpha_1$-casein (f 1-23) and their ability to degrade
individual intact caseins. Strains found to represent distinct proteinase
enzymes within lactobacilli species will then be added to cheese slurries and
Mozzarella cheese to investigate the effect of these enzymes on proteolysis and cheese functionality. Knowing characteristics of starter culture proteolytic systems would provide a sound basis for starter culture selection; so that functionality can be customized to meet customer requirements. This information would then make it possible to develop starter culture systems for optimum manufacture of both full and lower fat Mozzarella cheese.

1. Significant Progress against Objectives:
Objective 1, Step 1 - Thirty three lactobacilli strains have been characterized for cell morphology, Gram stain and sugar production (API characterization), and are currently being evaluated for membrane fatty acid analysis. Twenty additional strains of lactobacilli have just been received and preliminary characterization has started. Forty strains of S. thermophilus have also been screened and characterized. These strains are currently being tested for proteinase activity levels.

Objective 1, Step 2 - Eighteen strains of lactobacilli (Lactobacillus helveticus and Lactobacillus delbruekii ssp. bulgaricus) have been characterized for proteolysis using the α₁-casein (f 1-23) method. Analysis for the remaining characterized strains is underway.

Objective 1, Step 4 - OPA analysis for total proteolysis has been started with the characterized strains.

2. Significant Conclusions:
Preliminary data for the α₁-casein (f 1-23) method indicates two possible types of proteolysis can be found in lactobacilli and differences in a preferential cleavage pattern of the α₁-casein (f 1-23) for various strains into the final peptide products can be used for strain characterization.

3. Anticipated Problems/Delays:
Many of the lactobacilli cultures at the Weber State Culture Collection were lost due to a freezer malfunction and it has taken some additional time to obtain new cultures.

Publications:
None at present.

Theses:
None
Published Abstract:
None at present.

Presentations:
None at present.

Patent/Invention Disclosures:

Technology Transfer Activities
For information on licensing contact:

Visitors Hosted:
None
Western Dairy Center
Project Report
Reporting Period January 1, 1998 — December 31, 1999

Principal Investigators: Donald J. McMahon, Utah State University
Co-Investigators: Craig J. Oberg, Weber State University
Jeff Broadbent, Utah State University
Rajiv Dave, South Dakota State University

Project Title: Understand the role of proteolysis on functional properties of Mozzarella cheese

Institution’s Project #: 5-48181

Project Completion Date: December 31, 2000

Establish knowledge matrices relating the effects of processing parameters on cheese functionality by 12/31/01: Understanding role of moisture and proteolysis on functional properties of cheese.

Modifications to Project/Budget:
The remaining parts of the project will be completed by Dr. Rajiv Dave who has accepted a faculty position at South Dakota State University.

Project Objectives: (Include any revisions to objectives)
1. Determine the contribution of alphas1-casein hydrolysis on melting properties of mozzarella cheese.
2. Determine the contribution of beta-casein hydrolysis on melting properties of mozzarella cheese.

Project Summary: (Suitable for inclusion in Center documents released to the public)
The purpose of this project is to develop an understanding of how proteolysis during storage of mozzarella cheese influences cheese melting properties. In particular, the proteolysis that occurs during storage of mozzarella cheese will be profiled so that changes in melt and stretch functionality can be correlated with breakdown of alphas1-casein and beta-casein into peptides.
The project is designed to extend the knowledge of cheese proteolysis in mozzarella cheese beyond that which is currently known by monitoring the disappearance of intact proteins during refrigerated storage, by making cheeses that purposely have increased hydrolysis of alphas1-casein or b-casein. To avoid the influence of starter culture bacterial enzymes on proteolysis, the cheese will be made using direct acidification (i.e. without any cultures being added to the milk). Various levels of chymosin and plasmin will be added
during cheesemaking to modify the extent of proteolysis of alphas1-casein and beta-casein, respectively. The extent of proteolysis, as measured by the disappearance of the intact proteins and the appearance of peptides, will be correlated with melting, rheological and functional properties of the cheese.

1. Significant Progress against Objectives:
The preliminary trials were successfully completed by the end of September 1998. Methodologies for the manufacture of cheeses and analyses of various rheological and bio-chemical parameters were standardized during this time. Trials for Objective 1, to study the role of alphas1-casein hydrolysis on meltability and rheological properties of fat-free, reduced fat and part-skim mozzarella cheeses have been completed. Mozzarella cheeses with 0, 10 and 20\% fat were made using direct acidification to test the influence of coagulant concentration on proteolysis, meltability and rheological properties of cheeses during 60 d storage periods at 4°C.

Nine batches of milk (at three fat levels) were acidified to pH 5.65 (using acetic/citric acid combination) and then coagulated with 0.2x, 1x, and 4x recombinant coagulant at 37, 35 and 32°C, respectively. After cutting and healing the curd, pH was further dropped by adding glucono-delta-lactone to the whey and the curd cooked to 44°C and salted. The curd was then hand stretched in 5% brine at 82°C, molded, cooled in cold water, cut into 4 pieces, vacuum packed and stored at 4°C. The cheeses were sampled for fat, protein, moisture, salt, and calcium analysis on d 1. Changes in pH, meltability (using a tube test in an oil bath at 90°C for 16 mins), extent of proteolysis (using an acid-urea capillary electrophoresis and 12.5\% TCA-soluble nitrogen) and rheological properties (stress sweep and temperature sweep tests) were monitored at 1, 15, 30 and 60 d.

The first set of trials for Objective 2 were conducted during April to June, 1999. However, it was observed when the extent of proteolysis was determined that adding plasmin had no significant effect. Either the added plasmin was lost in the whey or inactivated during cheese making. A different experimental design from that originally proposed is to be conducted at South Dakota State University to study the influence of beta-casein hydrolysis on melting properties of cheese.

2. Significant Conclusions:
Lowering fat content reduced the initial (d 1) meltability of the cheeses: 15.7, 13.8 and 7.8 cm of melt for the 20, 10 and 0\% fat cheeses made using 11x coagulant. Lowering the coagulant level to 0.2x reduced d 1 meltability: 9.1, 8.5 and 6.3 cm, respectively, with the greatest decrease occurring with the 20\% fat cheese. During storage, meltability increased for the 0\% fat cheese, bu
decreased for the 20% fat cheese after 15 d because increased stickiness of the cheese reduced flowability of the cheese in the glass tubes.

The time required to hydrolyze all intact alphaS1-casein was about 15, 30 and 60 d for cheeses made using 4x, 1x and 0.2x coagulant, respectively. The higher the fat content, the softer the cheese, i.e. the cheese had lower complex modulus yield values. Also, during aging, the cheeses became softer but aging of the cheese had less effect than fat content of the cheese. While increasing the chymosin content had a large impact on level of proteolysis, there was an increase in hydrolysis of beta-casein as well as alphaS1-casein.

3. Anticipated Problems/Delays:
The project will not be completed until December 31, 2000 with the final project work being conducted at South Dakota State University by Dr. Dave

Publications:
Nil

Theses:
Nil

Published Abstract:

Presentations:

Patent/Invention Disclosures:

Technology Transfer Activities
For information on licensing contact:
Not applicable at this time.

Visitorrs Hosted:

13
Principal Investigators: Dr. Jeffery Broadbent, Utah State University
Co-Investigators: Donald J. McMahon, Utah State University
Dr. Craig Oberg, Weber State University

Project Title: Application of a metabolic control switch to improve and control starter proteolysis in Mozzarella cheese

Institutions Project #: 98091

Project Completion Date: 12-31-00

National Research Plan (1997): Priority: 1; Goal: 4; Tactic: 2
Develop knowledge matrix database to improve the performance of cheese starters by using genetic and microbial technologies to select, modify, or adapt starter cultures.

Modifications to Project/Budget:
None

Project Objectives: (Include any revisions to objectives)
Objective 1: Characterize the Lactobacillus helveticus LH212 groESL heat shock gene promoter.
Objective 2: Construct an expression vector that incorporates the LH2112 groESL promoter.
Objective 3: Develop a model system to study temperature-dependent proteinase expression in Lactobacillus helveticus and Lactococcus lactis using the groESL promoter-based expression vector.

Project Summary: (Suitable for inclusion in Center documents released to the public)
The purpose of this project is to develop an expression system for important dairy starter cultures that will allow these bacteria to produce high levels of a desired protein during a specific (and transient) stage of cheese manufacture. Although such an expression system would have application in several dairy processes, research outlined in this proposal will focus on its use to avoid pitfalls presently associated with the addition of proteolytic enzymes to cheeses. To accomplish this, we are investigating: (1) the regulation of a tightly controlled Lactobacillus helveticus heat shock gene promoter; (2) application of this promoter in a gene expression system that can transiently induce enzyme production during the cook step in cheese manufacture; and (3) this system's performance in cheese using a model enzyme such as the Bacillus subtilis neutral protease.
experiments will allow us to determine whether a natural metabolic switch can be employed to avoid problems presently associated with the addition of proteolytic enzymes to cheese. Results from this work will provide basic and applied information needed by industry to control and accelerate functionality in Mozzarella cheese.

1. Significant Progress against Objectives:
Nucleotide sequence analysis of the *L. helveticus* LH212 groESL operon and Northern hybridization with a groEL DNA probe confirmed that the groESL promoter is tightly regulated at the transcriptional level by heat shock. Constitutive expression of groESL is very low in *L. helveticus* LH212 but transcription of the operon is induced more than 400% upon temperature upshift from 37°C to 52°C. To characterize the utility of this promoter, we designed PCR primers that would allow us to insert the promoter upstream of the *E. coli* gusA gene in the lactic expression vector pNZ272. This work is important because it will allow us to accurately quantify promoter activity in both *L. helveticus* and *Lactococcus lactis*. Unfortunately, our efforts to quantify promoter expression have been slowed by the discovery that this construct apparently is lethal in *E. coli* hosts. All of the clones we have isolated to date do not display any GusA activity and DNA sequence analysis of the clones has shown that they all contain mutations that abolish gusA translation. Since GusA expression from pNZ272 has been previously demonstrated in *E. coli*, we believe that the inability of this host to support our construct may be due to very high constitutive gusA expression from the LH212 promoter. To overcome this problem, we have now begun to perform cloning experiments in *Lactococcus lactis*, where constitutive expression will not occur due to this bacterium's ability to recognize the heat shock negative regulatory elements (CIRCE) on the LH212 promoter.

As part of our effort to study the LH212 promoter, we have also designed PCR primers that will allow us to investigate the influence of a UUG versus AUG initiation codon on gusA expression in lactic acid bacteria. Our interest in this topic stems from the finding that the *L. helveticus* groES gene, like its counterparts in *L. zeae*, *B. subtilis*, and *L. johnsonii*, utilizes the uncommon start codon UUG. The importance of UUG and other rare initiation codons in the genus *Lactobacillus* is unknown, but in *E. coli* and (to a lesser extent) *B. subtilis*, non-AUG initiation codons act to limit the expression of a gene product at the translational level. For this reason, our effort to study the role of UUG and other rare initiation codons in lactobacilli and lactococci will facilitate efforts to use the *L. helveticus* groESL promoter as a metabolic control switch.

2. Significant Conclusions:
Results to date support our hypothesis that the *L. helveticus* LH212 groESL promoter is a strong metabolic control switch that may be well suited for the development of a process-regulated gene expression system to control enzyme production in cheese starter bacteria.

3. Anticipated Problems/Delays:
The laboratory technician working on this project is expecting a baby in mid-March and will be out for 6 weeks on maternity leave.
Publications:


Theses:
none.

Published Abstract:

Presentations:


Patent/Invention Disclosures:
none

Technology Transfer Activities

For information on licensing contact:
Jeff Broadbent or Carl Brothersen

Visitors Hosted:
none
Western Dairy Center
Project Report
Reporting Period June 1, 1997 — Dec. 31, 1999

Principal Investigators: Dr. Jeffery Broadbent, Utah State University
Co-Investigators: Donald J. McMahon, Utah State University
Dr. Craig Oberg, Weber State University

Project Title: Improvement of Mozzarella Cheese Functionality by Understanding Exopolysaccharide Production in Thermophilic Starter Cultures

Institution’s Project #: 97079

Project Completion Date: 6-30-00

National Research Plan (1997): Priority: 4; Goal: 4; Tactic: 1
Understand the impact of polysaccharide production by starter cultures in cheese quality and functionality.

Modifications to Project/Budget:
None

Project Objectives: (Include any revisions to objective)
Understand the influence of microbial exopolysaccharides on Mozzarella cheese moisture status and the relationship of moisture to cheese composition and functionality.

Objective 1: Characterize the structure, molecular weight, and polymer properties of the exopolysaccharide produced by S. thermophilus MR-1C.

Objective 2: Isolate and characterize the S. thermophilus MR-1C gene cluster for exopolysaccharide production.

Objective 3: Transform EPS-Mozzarella starter cultures with the cloned gene cluster and evaluate the influence of these constructs on moisture level and functional properties of low-fat Mozzarella cheese.

Project Summary: (Suitable for inclusion in Center documents released to the public)
Mozzarella cheese functionality is significantly affected by cheese moisture level, and increasing moisture content has been used to improve the melting properties of low-fat Mozzarella cheese. Previous work by our group has shown that exopolysaccharide (EPS)-producing starter cultures can
be used to significantly increase the moisture content of lower fat Mozzarella cheese. We have identified a culture, Streptococcus thermophilus MR-1C, that produces a large capsular EPS, and have shown that this capsule is involved in cheese moisture retention. Ongoing experiments are characterizing the structure of the MR-1C EPS and the genes that encode its biosynthesis. This project will provide basic information needed to understand the influence of EPS on cheese quality and functionality.

1. Significant Progress against Objectives:

Our research confirmed that the MR-1C EPS has a novel basic repeating unit composed of galactose, rhamnose and fucose. Ongoing efforts to elucidate the precise structure of the MR-1C EPS have identified 16 or 17 different monosaccharides from NMR spectra. We have also completed nucleotide sequence analysis for a region encompassing 36 kb of the MR-1C chromosome that contains the cps gene cluster. The structural complexity of the MR-1C capsule is reflected in the fact that capsule production in this bacterium appears to involve at least 19 different genes. Like each of these EPS-related gene clusters characterized to date in S. thermophilus, the MR-1C cps genes lie immediately downstream of the deoD gene. The first 4 genes, cpsA-D, are highly conserved and are thought to function in regulation (cpsA), polymerization (cpsC), and membrane translocation (cpsD) during polysaccharide synthesis. The MR-1C cpsE gene encodes a protein that is 99% identical to the S. thermophilus Sfi6 EpsE protein, a phosphogalactosyltransferase that catalyzes the first step in EPS biosynthesis. The MR-1C cpsF gene product is also closely related (97% identical) to its Sfi6 counterpart, EpsF, which is believed to function as a branching galactosyltransferase. The similarity between these enzymes may be misleading, however, because DNA sequence analysis suggests that the MR-1C CpsF protein, like that of S. thermophilus CNRZ368, includes 50 amino terminal residues that are not predicted to occur in the Sfi6 EpsF enzyme. The MR-1C cps region downstream of cpsF is closely related to the eps gene cluster of S. thermophilus CNRZ368. However, the latter bacterium does not produce any detectable EPS, and our sequence data suggest this observation is likely due to frameshift mutations in CNRZ368 epsF and epsN genes.

Protein homology studies using deduced amino acid sequences from each of the 19 putative MR-1C cps genes has identified genes whose products may function as glycosyltransferases in the assembly of the repeating unit, regulation of Cps expression, and in polymerization and membrane translocation of the basic repeating unit. With the possible exception of cpsE, however, no specific function can be assigned to any of the MR-1C cps genes, and the role of many of these genes in capsule biosynthesis is entirely speculative. In addition, some of the cps genes we have identified could have general housekeeping functions and would therefore be present in both EPS- and EPS+ bacteria. Given the complexity and size of the MR-1C cps gene region, our goal to construct capsule-producing variants from fast acid-producing S. thermophilus starters will have to wait until we can identify
which MR-1C cps genes are both unique to this bacterium and essential or capsule production. This work is now underway.

Research was also performed to address an industry concern related to the use of EPS+ cultures in cheese. Several processors expressed a concern that EPS+ cultures may increase whey viscosity, and thereby retard the efficiency of whey concentration and drying. In response, we investigated the effect of capsular and ropy S. thermophilus starter bacteria on Mozzarella cheese and whey. Cheeses were manufactured on three separate occasions using Lactobacillus helveticus LH100 paired with one of four S. thermophilus strains: MR-1C (capsular EPS+), 360 (ropy EPS+), TAO61 (EPS commercial starter) and an EPS- mutant of MR-1C (DM10). As expected, cheese moisture levels were significantly (P < 0.05) higher in Mozzarella made with EPS+ versus EPS- cocci. Viscosity measurements of cheese whey that had been concentrated 5-fold by ultrafiltration, however, showed that whey from cheese made with S. thermophilus 360 was significantly more viscous (F < 0.001) than whey from cheeses made with MR-1C, TAC61, or DM10. No significant differences were noted in the viscosity of concentrated whey from cheeses made with S. thermophilus MR-1C, TAO61, or DM10. These data indicate that encapsulated (but not ropy) EPS+ S. thermophilus can be used to increase cheese moisture levels without affecting whey viscosity.

2. Significant Conclusions:
Our data demonstrate that the S. thermophilus MR-1C EPS is a novel polymer with unique and significant commercial applications.

3. Anticipated Problems/Delays:
none

Publications:

Theses:
Published Abstract:


Presentations:


Patent/Invention Disclosures:
none

Technology Transfer Activities
Confidentiality and material transfer agreements have been signed by several culture manufacturing companies who are evaluating them for commercial production.
For information on licensing contact:
Jeff Broadbent or Carl Brothersen
Western Dairy Center
Project Report
Reporting Period January 1, 1999 - December 31, 1999

Principal Investigators: Conly Hansen, Utah State University
Co-Investigators: Donald J. McMahon, Utah State University

Project Title: Controlling Chemical Composition and Functionality of Cheese

Institution’s Project #: 99204

Project Completion Date: 12/31/01

National Research Plan (1997): Priority: C-13 B; Goal: Tactic:

Modifications to Project/Budget:
None

Project Objectives: (Include any revisions to objectives)

To determine the influence of pH, calcium, salt and moisture content of cheese on shredability and meltability.

Objective 1: To develop a high pressure injection system for modifying the chemical composition of cheese.

Objective 2: To modify pH, calcium, and salt contents of cheese while keeping all other parameters constant, and determine their influence on functionality.

Objective 3: To determine the combinations of calcium, salt, and pH required for optimum shredding and melting of cheese.

Project Summary: (Suitable for inclusion in Center documents released to the public)

1. Significant progress against objectives:

High pressure injection is the process by which fluids are accelerated to high velocities by passing through nozzles at pressures of 800 to 85,000 psi (5.5 to 34.5 Mpa) and injected into soft foods. High pressure systems have been developed at Utah State University with nozzle sizes from 4/1000 to 10/1000 inch (.1016 to .254 mm) in diameter. These high pressure systems have all featured electronic on and off control of the high pressure stream. More recently, it
1. **Significant progress against objectives:**

High pressure injection is the process by which fluids are accelerated to high velocities by passing through nozzles at pressures of 800 to 5,000 psi (5.5 to 34.5 Mpa) and injected into soft foods. High pressure systems have been developed at Utah State University with nozzle sizes from 4/1000 to 10/1000 inch (.1016 to .254 mm) in diameter. These high pressure systems have all featured electronic on and off control of the high pressure stream. More recently, it became evident that the heads should have multiple rows of nozzles. Multiple rows greatly reduce the time needed to completely inject a cheese sample and insure more uniformity between injections. It also became evident that high pressure heads needed to be more easily cleanable. Too much time was being spent to remove nozzles and completely disassemble heads to clean a single nozzle. A new head was designed based on initial tests with plastic models to determine designs that provide tangential flow across the nozzles and uniformity of force from all nozzles. This head has been built and tested and found to perform extremely well. The head was installed on our injection table apparatus which features a conveyor belt electronically controlled to inject at variable intervals, pressures and durations. Relatively large blocks of cheese can be injected with this apparatus in a matter of seconds. See Figure 1.

![Figure 1. Multi-row injection head for large blocks of cheese.](image)

Much of our work thus far has been with small quantities of cheese ≤ 1 lb (454 g). A relatively small head was modified to fit 10 working nozzles, 0.006" in diameter. A small homogenizer pump is capable of powering this head which was also mounted above a conveyor belt system. This device has been of tremendous help as we had been injecting one nozzle at a time and moving the cheese beneath the nozzle by hand. The advantage of this system over the larger system is in convenience for small injection jobs and that we only need prepare about 2 quarts of injectate for a given run. In the coming year, we plan to add controls to the conveyor to electronically control injection interval and burst duration.
Injection of Colored Water

The first experiments performed with the high pressure system was to inject colored water only into the cheese. The effects of injection time and pressure on injected cheese weight gain, depth of injection and solids lost with injectate that passed through the cheese were evaluated. Full fat Mozzarella cheese (approximately 45% moisture) manufactured in the Utah State University Dairy Production Laboratory (Logan, UT) was injected with water colored with FD&C Blue #1 Powder (Chr. Hansen Inc., Milwaukee, WI). All cheese was from the same block and between 12 and 19 days old at the time of injection. The cheese was injected for three time periods, 1, 2, and 3 seconds, and three pressures, 1,000, 1,500, and 2,000 psig (6.9, 10.3, and 13.8 MPa). Injections were performed at each time period for the three pressures so that each replication consisted of nine injections. Analysis of variance indicated that there was no significant difference between replications for any of the response variables (data not shown).

Cheese blocks were injected in duplicate. Replicate cheese blocks and some trials within replicates were injected on different days. All cheese blocks were injected 16 times on one side using a single orifice nozzle. The injections began approximately 0.4" (10 mm) from the edge of the cheese and were arranged in a 0.4 x 0.4 inch (10 x 10 mm) grid pattern. The injection orifice was 0.006 in (0.1524 mm) and the cheese was positioned approximately 0.6 in (15 mm) from the orifice during injection. Cheese weight was recorded immediately before and after injection. Cheese blocks starting weights was approximately 10.2 Oz (290 grams) but varied between 8.5 to 12.6 Oz (240.7 to 357.5 g). The injectant that was not incorporated into the cheese block was collected and weighed. This injectant was also dried to determine its total solids content (solid particles from the cheese). Injected cheese blocks were weighed then vacuum packaged and stored at least 24 hours prior to cutting to determine injection depth. Depth was determined by observing the dye in the cheese and measuring the penetration depth of the injected dye.

The results for weight increase showed that as time and pressure increased, the weight gain of the cheese increased. Three way analysis of variance showed that the increase in pressure did not significantly change the weight gain in cheese but the increase in time from 1 to 2 seconds significantly increased the weight of the cheese (P = 0.10, P = 0.017, respectively) (Table 1). The actual increase in weight was very small, less than one percent in every case.

Table 1. Weight gain in Mozzarella cheese injected at various pressures and injection times using 0.2 mm (0.008 in) nozzles.

<table>
<thead>
<tr>
<th>Pressure (MPa)</th>
<th>Mean weight gain*</th>
<th>Time (sec)</th>
<th>Mean weight gain**</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.9</td>
<td>0.41*</td>
<td>1</td>
<td>0.32*</td>
</tr>
<tr>
<td>10.3</td>
<td>0.60*</td>
<td>2</td>
<td>0.60b</td>
</tr>
<tr>
<td>13.8</td>
<td>0.61*</td>
<td>3</td>
<td>0.69b</td>
</tr>
</tbody>
</table>

*Means with different superscripts are different (P < 0.05) (no differences indicated).
**Means with different superscripts are different (P < 0.02).

The quantity of injectant that passed through the cheese block increased significantly as time was increased from one to two and from two to three seconds (P < 0.001). It also increased as pressure was increased from 6.9 to 10.3 MPa (P < 0.003) (Table 2).
Table 2. Injectant loss in Mozzarella cheese injected at various pressures and injection times using 0.2 mm (0.008 in) nozzles.

<table>
<thead>
<tr>
<th>Pressure (MPa)</th>
<th>Mean injectant loss*</th>
<th>Time (sec)</th>
<th>Mean injectant loss**</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.9</td>
<td>26.52g*</td>
<td>1</td>
<td>9.572g*</td>
</tr>
<tr>
<td>10.3</td>
<td>32.91g*</td>
<td>2</td>
<td>33.192g*</td>
</tr>
<tr>
<td>13.8</td>
<td>38.61g*</td>
<td>3</td>
<td>55.277g*</td>
</tr>
</tbody>
</table>

*Means with different superscripts are different (P<0.003).
**Means with different superscripts are different (P<0.001).

Solids analysis on lost injectant showed no significant difference at the 5% level for time or pressure changes. Solids levels at injection pressures of 6.9 MPa were significantly lower than those from other injection pressures. This may indicate that the cheese matrix is less disrupted by injection at this lower pressure. The lack of deep penetration shown by the depth analysis supports this reasoning.

The depth of injection was significantly altered by the injection pressure. Each increase in pressure resulted in a significant increase in depth (P<0.008). Increasing time also resulted in an increase in depth (P<0.02), however, the time increment from 2 to 3 seconds was not shown to be significant. This time increment may show no significance because at injection times of 3 seconds, injectant often passed through the cheese. Its significance may be underestimated by these results. The lack of significance shown in the weight gain results for this time interval may also be explained by this reasoning. Table 3. shows the means of depth for various pressures and times.

Table 3. Means of depths of injection in Mozzarella cheese injected at various pressures and injection times using 0.2 mm (0.008 in) nozzles.

<table>
<thead>
<tr>
<th>Pressure PSI (MPa)</th>
<th>Mean depth of injection* (inches)</th>
<th>Time of injection (sec)</th>
<th>Mean depth of injection** (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000 (6.9)</td>
<td>1.8* (4.50)</td>
<td>1</td>
<td>1.8* (4.57)</td>
</tr>
<tr>
<td>1500 (10.3)</td>
<td>2.65b (6.72)</td>
<td>2</td>
<td>2.7b (6.85)</td>
</tr>
<tr>
<td>2000 (13.8)</td>
<td>3.15b (8.00)</td>
<td>3</td>
<td>3.06b (7.8)</td>
</tr>
</tbody>
</table>

*Means with different superscripts are different (P<0.008).
**Means with different superscripts are different (P<0.02).

Injection of Calcium

Calcium chloride was successfully injected into Cheddar cheese. Both the ash percentage and the calcium content of the injected cheese was greater than the control (P<0.001). High pressure injection resulted in a uniform increase in ash content in the cheese, as indicated by comparing the ash content of the injected surfaces to the center portion of the cheese. Calcium concentrations
also increased uniformly in injected cheese blocks. There was no significant difference between different sections of the injected cheese blocks.

Commercially produced Cheddar cheese was obtained locally in a 2 lb (907 kg) loaf. The loaf was divided into three blocks approximately equal in size and a smaller control block. Except for injection, the control block was treated and stored the same as the injected blocks. Anhydrous calcium chloride was purchased from Mallinckrodt Chemical, Inc. (Paris, KY), and dissolved in water to form a 30% (W/W) solution. This solution was injected into the cheese using a high pressure injection apparatus manufactured at the Utah State University Food Engineering Lab. The cheese was injected using a single orifice injector repeatedly so that a grid of injections was formed on the top and bottom of the cheese blocks in a 0.4 x 0.4 inch (1 x 1 cm) configuration. The orifice diameter of the injection nozzle was 0.008 in (.2 mm) in diameter. Injectate was pumped to the orifice at high pressures, 2,000 psi (13,800 kPa). Cheese blocks were 3.54 x 3.54 x 1.97 inch (9 x 9 x 5 cm). Cheese blocks increased in weight due to injection approximately 1%. Three cheese blocks were injected and one block served as a control. The cheese was allowed to equilibrate 21 days before sampling.

Injected cheese blocks were divided into thirds perpendicular to the direction of injection by cutting the blocks into top, middle, and bottom sections. The control cheese block was not divided into thirds but was sampled as described hereafter. The cheese was sampled by cutting each section of injected cheese parallel to the direction of injection with a cylindrical cutter, 1.6" (4 cm) in diameter. Control cheese was also sampled with the cylindrical cutter. Each cylinder of cheese as approximately 1.6" (4 cm) tall. The cylinder of cheese was cut into quarters and the quarters were trimmed down to approximately 0.35 Oz (10 g) portions. These 0.35 Oz (10 g) portions were dried at 212°F (100°C) in porcelain crucibles in a VWR Scientific Inc. drying oven (Model 1640, Sheldon Manufacturing Inc., Cornelius, OR), heated on a hot plate (Corning, Model PC - 500) until they stopped smoking (approximately 24 h), and ashed in a muffle furnace (TEMCO Thermoelectric Mfg. Co., Model F1730, Dubuque, IA). The ashed samples were weighed and the percent ash was determined.

Samples for calcium analysis were prepared according to the procedure described above. Samples were taken to the Utah State University Testing Labs (Logan, UT) where calcium analysis was performed.

One way analysis of variance was performed using Minitab Statistical software (Minitab Inc., State College, PA) to determine differences between the top, middle, and bottom sections of the cheese blocks as well as differences between different cheese blocks. Minimum significant difference calculations were performed according to the procedure described by Moore and McCabe (1993).

Calcium determinations showed an increase of approximately 0.2 percentage units (a 0.25% increase in calcium). The average percent calcium in the control and injected cheese blocks are shown in Table 4. There was no significant difference between the injected cheese blocks in terms of calcium content. There was also no significant difference in calcium content between the top, middle, and bottom of the injected cheese blocks. It was expected that the middle of the cheese block could contain less calcium than the outside of the cheese block. This uniformity may indicate that the injection of calcium chloride by this method produces a uniform distribution of calcium in cheese, or that calcium can migrate within the block of cheese and achieve a uniform distribution.
Table 4. Average Calcium Content

<table>
<thead>
<tr>
<th>BLOCK</th>
<th>N*</th>
<th>AVERAGE (%)</th>
<th>STD. DEVIATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>0.79</td>
<td>0.04</td>
</tr>
<tr>
<td>Injected</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Block 1</td>
<td>12</td>
<td>1.05</td>
<td>0.08</td>
</tr>
<tr>
<td>Block 2</td>
<td>12</td>
<td>1.03</td>
<td>0.08</td>
</tr>
<tr>
<td>Block 3</td>
<td>12</td>
<td>1.02</td>
<td>0.08</td>
</tr>
</tbody>
</table>

*Number of sampling units per block

*bMeans with different superscripts are different (P<0.001)

2. Significant Conclusions:

1. Cheese can be injected using the high pressure injection apparatus. Injection may be performed for a variety of purposes. The parameters of injection studied indicate that depth, weight gain, and injectant loss can be increased by increasing pressure or time. Some solid material is lost from the cheese during injection. There are fewer losses associated with injection at relatively low pressures but these pressures do not allow deep injectant penetration or promote "large" weight gains. Depending on the desired outcome and the textural properties of the cheese to be injected, optimal conditions can be established to maximize depth and weight gain and minimize injectant and cheese loss.

2. Calcium can be successfully injected into cheese using a high pressure injection system. Additional research is being performed to obtain information on the, chemical, and physical effects of injecting CaCl₂ into cheese.

3. Anticipated Problems/Delays:

Publications:

Theses:

Published Abstract:

A.J. PASTORINO, N. Ricks, C. Hansen and D.J. McMahon. Effect of Water and Calcium Injection on the Microstructure, Physical and Functional Properties of Mozzarella Cheese. Accepted for presentation at the IFT 2000 Annual Meeting & Food Expo, June 10 - 14, 2000, Dallas, TX
Western Dairy Center
Project Report
Reporting Period July 1, 1997 — December 31, 1999

Principal Investigators: Dr. Bart Weimer
Co-Investigators: 

Project Title: Identification and characterization of components of the proteolytic enzyme system of Lactobacillus helveticus that effect bioactive peptide accumulation, Utah State University part.

Institution’s Project #: 97083
Project Completion Date: 6-30-00


Modifications to Project/Budget:
None

Project Objectives: (Include any revisions to objectives)
1. To screen strains of Lactobacillus helveticus for the type and level of bioactive peptides/bioactive peptide precursors which accumulate as the result of the organisms growth in milk. 2. Determine which components of the proteolytic systems of the selected strains of L. helveticus are essential for the accumulation of the bioactive peptides/bioactive peptide precursors from milk. 3. Construct strains of L. helveticus, which accumulate elevated levels of the bioactive peptides/bioactive peptide precursors of interest.

Project Summary: (Suitable for inclusion in Center documents released to the public)
Strains of lactobacilli (60) were screened for degradation of the alpha-casein 1–23. In Lactobacillus helveticus at least 5 patterns were found. Specific peptides are unique to strains. Each peptide profile is being investigated to determine unique peptides in each strain. Whole caseins are being screened for production of peptides with HPLC/MS. Knockout mutants provided by Jim Steele were screened for peptide production from alpha-casein 1–23, and found that Lb. helveticus CNRZ32 produces at least 2 proteases and that the peptide pattern is a product of those enzymes. Pure peptides with bioactivity have been synthesized and were as standards for further analysis of proteolytic patterns. The gene sequence is partially determined, which will be combined with the hydrolysis patterns to form a classification system. Accumulation of peptides is being determined as the hydrolysis progresses.
Hydrolysis of the alpha-casein 1-23 fragment by lactobacilli was complete within 15 minutes of incubation at 37°C. Identity of the resulting peptides were determined for CNRZ32 and protease deficient mutants by HPLC/MS and co-elution of synthetic peptide standards. Multiple peaks were observed with 11 dominate peptides being produced in the wildtype. The most noteworthy is the 1-9 fragment that was produced at high levels in all strains tested. It appears that the substrate is hydrolyzed once in various locations that result in two fragments, opposed to hydrolysis of each fragment in multiple locations. For example, one fragment pair was 1-9 and 10-23. In some cases, one part of the pair was not observed. However, since every peak was not sequenced, it is possible that it was present and not identified. Alternatively, these peptides may have been metabolized by cultures during incubation, but this is unlikely considering the short incubation times used in the assay. These data are ready to be used to define a protease classification system.

The proteolytic system of *L. helveticus* is significantly different to that of lactococci. This strain contains at least 2 proteases. The first cloned and sequenced has a different substrate binding site and different substrate specificity to that of the lactococcal proteinase. The new proteinase changed the peptide pattern. In the deletion mutant, 3 bitter peptides were not produced, despite the large amount of 1–9 produced by all strains tested (>65 cultures).

1. Significant Progress against Objectives:
   All objectives are being completed as listed in the proposal.

2. Significant Conclusions:
   Numerous hydrolysis patterns are found in the strains. At least 2 proteases seems be produced in *L. helveticus* CNRZ32. The gene sequence is finished, and was found to be a new type of enzyme in LAB. *L. helveticus* and *L. casei* produce similar degradation patterns from the 1-23 fragment. Other lactobacilli are significantly different from these two species.

3. Anticipated Problems/Delays:
   A new student just arrived to finish the project.

Publications:


Theses:
Paul Joseph – Ph.D. candidate

Published Abstract:

Presentations:


Patent/Invention Disclosures:
none

Technology Transfer Activities
For information on licensing contact:
Bart Weimer
Milkbugs@cc.usu.edu
435 797 3356

Visitors Hosted:

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Western Dairy Center
Project Report
Reporting Period July 1, 1999 — December 31, 1999

Principal Investigators: Bart Weimer, Utah State University
Co-Investigators: Lan-Szu Chou, Utah State University

Project Title: The proteome of lactic acid bacteria

Institution’s Project #: 99208

Project Completion Date: 6/30/00

National Research Plan (1997): Priority: 4; Goal: 4; Tactic: 1

Modifications to Project/Budget:
None

Project Objectives: (Include any revisions to objectives)

Hypothesis
The growth environment impacts the expression and interaction of intracellular proteins which change the phenotypic characteristics of lactic acid bacteria which impacts their ability to survive and metabolize for extended periods.

Objectives
1. Obtain access to the genome sequence database for Lactobacillus acidophilus that is being determined by CalPoly (available in October 1999)

2. Determine which proteins are expressed during optimal growth.

3. Determine which proteins are expressed during stress (sugar starvation, heat shock, cold shock, pH, and bile).

4. Determine which proteins are expressed during incubation in gut-like conditions during extended incubation with L. acidophilus. Metabolic end products will also be determined during these experiments.

5. Compare the genome sequence with the protein expression patterns to determine the influence of environment and intracellular protein on the survival and phenotype of these bacteria.
Western Dairy Center
Project Report
Reporting Period January 1, 1999 — December 31, 1999

Principal Investigators: Dr. DeLoy Hendricks, Utah State University
Co-Investigators:

Project Title: Evaluation of the effect of concentrated buttermilk on iron transport and absorption.

Institution’s Project #: 99205
Project Completion Date: June 30, 2000

| National Research Plan (1997): | Priority: 4; Goal: 4; Tactic: 1

Modifications to Project/Budget:
None on Budget

But tests have shown the CaCO₂ cell culture does work. However, the milk fractions portion was inconclusive. Therefore, we will use fresh human, goat and cow’s milk to determine their respective effects on CaCO₂ cell transport and uptake of iron and zinc.

Project Objectives: (Include any revisions to objectives)

To evaluate the effect of concentrated buttermilk fractions on iron transport and absorption using the CACO₂ cell line.

Project Summary: (Suitable for inclusion in Center documents released to the public)

Sweet buttermilk was treated by molecular sieving (MS) or fermentation (FM) to remove the lactose in order to concentrate potentially bioactive components. In the final dried products, MS buttermilk contained 35% carbohydrate while the FM buttermilk contained only 12.5% carbohydrate. CACO₂ cells were grown to confluence on transwell plates which allow membrane transport from an upper chamber to a lower chamber across the cell membranes.

Cells which had been grown to confluence in a 10% Fetal Bovine Serum (FBS) media (Iron replete) transported 59 Fe at only 2/3 the rate of cells grown in a media containing no FBS (Iron depleted). Iron transport was enhanced by both buttermilk treatments at 1, 2 and 3% dilution when added to the media at 1%. However, transport response did not correlate with treatment dilution. Iron transport in Iron deplete cells was highly variable while
transport by Iron replete cells was consistently enhanced over 40% by MS and 120% by FM. A Ganglioside standard enhanced Iron transport by 100% while a Sphingomyelin standard increased Iron transport by 86%. It appears in this model system that some components of the buttermilk concentrates, perhaps Sphingomyelin or Gangliosides facilitate Iron transport across cell membranes of Iron replete cells.

1. Significant progress against objectives:
Both bacterial fermentations and molecular senine of buttermilk was effective in reducing lactose concentration thus concentrating potentially bioactive components of sweet cream buttermilk. Either method is commercially feasible.

Iron transport by CACO₂ cells has been shown to be responsive to both buttermilk concentrates and to standards of sphingomyelin and ganglioside. However, because the increase in Iron transport was not linearly correlated with buttermilk concentration, additional studies must be conducted. Also, the failure to get consistent treatment responses in the Iron deplete CACO₂ System raises further questions as to the mechanism of action.

In future studies, Fetal Bovine Serum should be left out of the media because of the varied factors it may be contributing to cell performance.

2. Significant Conclusions:
The CACO₂ cell model exhibits typical iron absorption characteristics in that Iron deprived cells absorb and transport more iron than Iron replete cells.

The buttermilk concentrates do enhance iron transport by CACO₂ cells. This enhancement or iron transport is much clearer in iron replete cells but is not dose responsive.

3. Anticipated Problems/Delays:

Publications:
None

Theses:
Western Dairy Center
Project Report
Reporting Period January 1, 1998 — December 31, 1999

Principal Investigators: Joseph Irudayaraj, Penn State University
Co-Investigators: Donald McMahon, Utah State University

Project Title: Process technology to improve the flavor of heated milk
Institution’s Project #: 98101

Project Completion Date: June 30, 2000

National Research Plan (1997): Priority: Goal: Tactic:
Fluid Milk 3.3: Exploration of alternate processes for specific products and development of ingredients and beverages

Modifications to Project/Budget:
The total request is the same. Due to the change in the institution of the PI a modification was proposed and presented to the DMI through the Western Dairy Center.
The heating conditions for the conventional and Electroheating technology was refined to reflect the actual conditions. No major change was made.

Project Objectives: (Include any revisions to objectives)
1) Determine the effect of electroheating on flavor and sensory attributes
2) Compare the flavor characteristics to conventional UHT processes
Objectives for Year 1 is to conduct preliminary trials and obtain preliminary data
on flavor, sensory, and chemical attributes.

Project Summary: (Suitable for inclusion in Center documents released to the public)
Raw milk was pasteurized using plates and steam heat exchangers at Utah State University’s dairy pilot plant. The pasteurized milk was then UHT treated using the Electroheating system developed by Raztek Corporation.
Two experimental trials were conducted at Raztek Corp. The initial trials were used to set up the process parameters in the Electroheating system for subsequent experiments. Temperatures considered were 135, 145, and 155 deg C. Holding times used were
0.5 and 4 secs. Preliminary volatile flavor tests were conducted and potential volatile compounds were identified. Training of the sensory panel is complete for subsequent sensory analysis. A controlled sensory analysis indicated that the cooked, sour, and stale flavor in the Electroheated samples were much lower than the commercial variety. The protein denaturation in Electroheated milk was less than half of the commercial variety tested.

1. Significant Progress against Objectives:
1) Preliminary trials are complete
2) Preliminary sensory, flavor, and chemical analysis results are obtained

2. Significant Conclusions:
1) There is a significant reduction in protein denaturation of Electroheated milk
2) Preliminary sensory analysis using the trained panel indicated a reduction in the cooked, sour, and stale flavor of Electroheat processed UHT milk.

3. Anticipated Problems/Delays:
All the chemical and flavor tests will have to be completed within the same week of the experiment. In addition the milk is being shipped to different destinations for analysis and experiments.
From the initial trials a protocol has been worked out. We hope to overcome this hurdle by a collaborative and coordinated effort.

Publications:
___none___

Theses:
___none___

Published Abstract:
___none___

Presentations:
___none___

Patent/Invention Disclosures:
PROCESS TECHNOLOGY TO IMPROVE FLAVOR OF HEATED MILK

Joseph Irudayaraj and Donald McMahon

Introduction

Ohmic heating is a novel technology currently utilized worldwide to produce a variety of high-quality, low and high-acid products. The most distinct advantages of ohmic heating are: its ability to accurately control the temperature of the product, uniform heating of liquids, fast heating rates, no fouling and scorching of product on the walls, low maintenance, no residual heat after the current is shut off, and very low heat losses.

The ohmic heater consists of a column which typically contains four electrode housings machined from solid blocks and encased in stainless-steel for mechanical integrity. The housings are connected together using stainless steel inter-connecting tubes lined with an electrically insulating plastic liner. The column is mounted in a vertical or near vertical position with the flow of product in an upward direction. Power to the heater is provided by a main step-up transformer with the power being controlled on the primary side of the transformer using a feed-forward temperature control system. A feed-back monitoring system is used to prevent any long term drift in outlet temperature (Skudder, 1991). One of the first applications of ohmic heating to food products was due to the work by deAlwis and Fryer (1990). However, only recently this technology has received considerable attention in thermal processing of foods (Qihua et al., 1993).

Commercial ohmic heating systems are already being used for continuous processing of food materials (Stirling, 1987; Skudder, 1991). However, such applications provide very less or almost no information on the design and performance aspects of ohmic systems (Biss et al., 1989; Stirling, 1987). An experimental ohmic heating unit was designed and fabricated for continuous thermal processing of liquid foods and applied for orange juice processing. The unit was supported by a data acquisition system for sensing the liquid temperature distribution, line voltage, and current with time. Experiments indicated that the temperature of liquid at the outlet was decided directly by the flow rate, electrical conductivity, applied voltage gradient and dimensions of the heating unit (Qihua, 1993). Only temperature and current data was collected for heating of orange juice.

The electroheating technology is a unique form of ohmic heating which is based on the principle of passing electric current through a food product which causes the food to heat. Applications and details of the general ohmic heating system is provided in this section. The fundamental principles have been discussed by Sastry (1992). The major benefit of the process is that heating occurs volumetrically and the product does not undergo large temperature variations. The ohmic heater was originally developed by the United Kingdom Electricity Research and Development Center at Capenhurst (UK). APV Baker obtained a license for the system in 1983, and since then, has substantially improved its design and applications. The applications initially dealt with are: 1) hygienic production of value added readily prepared meals, 2) pre-heating of food product prior to in-can sterilization, 3) pasteurization of particulate fruit products for hot-filling, and 4) aseptic processing of prepared meals. In 1988, APV Baker was awarded the “Food Processing Award” for technical innovation and in 1990 was awarded the “DuPont Award” and a “Du-Pont Diamond Award” for pioneering the development of this technology. This process, to our knowledge, has not yet been successfully applied to heat processing of milk. Parameters obtained from this work could be used in the future work in modeling, property determination and process optimization. In order to fully exploit the inherent advantages of a process such as electroheating, the quality of the processed product becomes important. A brief review of flavor and sensory characteristics are presented below.
**Effect of flavor**

Changes in the flavor of milk become more pronounced the higher the temperature used to process the milk. For example, many compounds have been identified in sterilized concentrated milks and in UHT processed milks, originating from both proteins and fat (Patel et al., 1962, Bassette & Jeon, 1983). Some of these include diacetyl, lactones, alcohol ketones, maltol, vanillin, benzaldehyde, acetophenone, etc. However, the exact compounds responsible for flavor are not yet known.

Immediately after processing, UHT-processed milk has a more-or-less strong 'heated' flavor with a sulfurous odor. This odor disappears rapidly and the heated flavor decreases with time and a characteristic 'UHT' flavor, described as 'stale' develops which becomes stronger with time. Badings and Neeter (1980) have identified 45 different compounds contributing to 'UHT' flavors, with an additional 12 unidentified compounds. A list of flavor compounds is also provided by Burton (1988). UHT processing allows milk to be stored at room temperature for 1 to 2 years without microbial spoilage. However, increased temperature treatments used in UHT processing cause cooked or caramelized flavor due to Maillard browning reactions. UHT milk processed using steam injection results in less cooked flavor compared to indirect heating, but does not eliminate this defect (Burton and Perkin, 1970). To further reduced cooked flavor, extend the shelf life beyond 14 days, and expand the capabilities to transport milk without spoilage, milk is being commercially processed at temperatures well above those used for pasteurization, more typical of those for UHT processing; and packaged under aseptic or ultra-clean conditions so as to produce extended shelf life (ESL) milk. No research has been performed on processing milk at extremely high heating rates for a very short time (faster than is obtainable with steam heating) using direct heating of the product by electrical current.

**Sensory Evaluation**

Sensory evaluation of milk is important for quality control. Classification of grades are based on defects and quantitative assessment of the terms "slight", "definite", and "pronounced". Different authors have published descriptors of off-flavors in milk and milk products (Bodyfelt et al., 1988; IDF, 1987). These descriptors are used for training of sensory panels and off-flavor recognition. Although, it is difficult to perceive a mixture of flavors, for some, threshold values have been published (Connolly et al., 1980; Jellinek, 1985). But less information is available on safety, sensory qualities, or absolute shelf life of milk stored for longer periods of time. Microbial survival, enzyme activity must also be considered when studying the characteristics of ESL products (Blake, et al, 1995).

The specific objectives of are to:

1) Determine the effect of electroheating on flavor characteristics of heated milk during storage, measured by both trained and untrained sensory panel.
2) Determine the effect of electroheating on volatile flavor compounds during storage and whey protein denaturation.

**Methods and Materials**

**Milk Processing**: Mixed herd raw milk standardized to 1% fat will be used in this study. The 1% fat content milk used in this study is classified as low fat milk (ie. fat content is less than 1.25%). Processing runs will be made in the USU Dairy plant (Utah State University) using a pilot plant system (Sterilab™, Alpha Laval system) equipped with a steam injection system (for direct heating) and a plate heat exchanger (for indirect heating). The capacity of the plant is 30 gal/hr and the preheating temperature range is...
between 70 and 90°C. The preheated milk will be heated to the desired temperature (in the range between 130 and 150°C) and flash cooled to 60°C. Processing temperature considered for ohmic heating are 135, 140, and 150°C. Holding times considered are 1 and 4 seconds.

**Protein Denaturation:** Whey protein denaturation will be measured using a modified procedure of Vakaleris and Price (1959). Ten milliliter milk samples are diluted with 40 ml distilled water. Twenty-five milliliter of this diluted solution is adjusted to pH 4.6 by drop wise addition of 0.1N HCl and filtered. From each filtrate, 10 ml is used to determine whey protein nitrogen (WPN) content by a semi-micro Kjeldahl procedure (Manji and Kakuda, 1987) using a Kjeltec Auto 1030 Analyzer (Tecator AB, Hoganas, Sweden). Percent whey protein denaturation is calculated using:

\[
\% \text{ Denaturation} = 100 \times \frac{WPN_{\text{raw milk}} - WPN_{\text{heated milk}}}{WPN_{\text{raw milk}}}
\]

**GC Analysis:** Flavor Chemistry

Samples for volatile analysis will be conducted using a dynamic headspace analysis (Brewer et al., 1997). A 50 g sample is placed into a 100-ml round-bottom flask fitted with a universal inlet adapter. Sample purge is conducted by immersing the flask in a 37°C circulating water bath. A submersible stirrer on highest setting and Teflon starburst stirring head (9.5 mm, Fisher Scientific, Pittsburgh, PA) is used to provide thorough agitation of samples, facilitate heat transfer, and increase gas/liquid interaction. Nitrogen purge gas is used to limit oxidative changes. Purge gas is passed through a moisture and hydrocarbon trap (Restek, Bellefonte, PA). Teflon tubing is used for all connections following the gas filters. Purge is conducted by swept surface to avoid foaming and inclusion of antifoam agents. Samples are purged at 800 ml/min for 20 min; final purge volume is 16 L.

Commercially available Carbopack (Supelco, Bellefonte, PA) traps are used with a bed weight of 350 mg. Following headspace sampling, the 50 mg breakthrough section of the trap is placed in a 2 ml vial and examined for analyte breakthrough. The 350 mg section is eluted with double distilled diethyl ether into a separate 2 ml vial then concentrated to ~100L.

Gas chromatography is carried out with a Hewlett Packard (Avondale, PA) 6890 gas chromatograph with flame ionization detector under the following conditions. Initial temperature is 40°C isothermic for 6 min; increased by 6°C/min to 210°C and maintained for 15 min. A Restek (Bellefonte, PA) Stabilwax-DA column is employed (30 m, 0.25 mm i.d., 1.0 µm film thickness). Column flow rate is 1.1 ml/min and sample size is 1.5 µl with splitless injection. HP ChemStation software is used for peak area determination and identification of recovered volatiles is obtained by comparison of analyte retention indices (RI) in an available database, by retention times of pure standards (Sigma-Aldrich, St. Louis, MO), and by mass spectroscopy.

Mass spectroscopy is performed with a Hewlett Packard mass spectrometer with column and chromatographic parameters as described above. Conditions are ion source temperature, 170°C; ionization voltage, 70 eV; mass scan range, m/e 20-300; scan rate, 1.0 scan/s. Compounds are identified in cheese samples by comparison of sample spectra with library reference spectra (NIST) and by comparing mass spectra and GC retention times to known standards. Quantification in cheese samples is based on the recovery of internal standards (20 l methyl butyrate solution) added to the milk at a final concentration of 0.010 g/g.

**Sensory Analysis:**
Trained panel

A trained panel was used to rate the intensity of taste and flavor attributes. The panel had been trained in September. There were 4 evaluation sessions. At each session the panels received 5 samples (control, T1-T4). The panelists did duplicate evaluations for each sample in each replication. The milk samples were served at 14.5°C.

Consumer evaluation

A consumer panel was used to rate consumers’ opinions about the milk samples. The panelists were screened for drinking low fat milk. Each consumer evaluated the 5 samples. For the consumer evaluations 40 persons evaluated samples from replication 1 and 39 persons evaluated samples from replication 2. There were 2 evaluation questions for each sample (see enclosed ballot). The values for the 9-point liking scale were 9=like extremely, 5=neither like nor dislike, and 1=dislike extremely.

Analysis

SAS General Linear Model (analysis of variance procedure) was used to analyze the trained and consumer data and Tukey’s to compare the means. Analysis of variance was used to analyze the trained and consumer data and Tukey’s to compare the means. The trained panel did duplicate evaluations for each set of samples from each rep. The data from the two reps were combined for the analysis of both the trained panel and consumer evaluations.

Results

Protein Denaturation:

A comparison of protein denaturation of electroheated milk with a commercial variety is given in Table 1. The electroheated milk processed at 145°C with minimum holding time had the least denaturation. Results were consistent with all experiments.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Sample ID</th>
<th>Trial 1 % Denaturation</th>
<th>Trial 2 % Denaturation</th>
<th>Trial 3 % Denaturation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Past. Milk 1</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>2</td>
<td>GS 2% fat</td>
<td>70.54</td>
<td>66.17</td>
<td>66.17</td>
</tr>
<tr>
<td>3</td>
<td>GS Skim milk</td>
<td>70.54</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Treatment1 (155, 1 sec)</td>
<td>23.65</td>
<td>28.65</td>
<td>28.91</td>
</tr>
<tr>
<td>5</td>
<td>Treatment2 (145, 1 sec)</td>
<td>21.04</td>
<td>21.1</td>
<td>21.11</td>
</tr>
<tr>
<td>6</td>
<td>Treatment3 (145, 4 sec)</td>
<td>35.26</td>
<td>36.95</td>
<td>36.95</td>
</tr>
<tr>
<td>7</td>
<td>Treatment4 (135, 4 sec)</td>
<td>27.35</td>
<td>30.21</td>
<td>30.18</td>
</tr>
</tbody>
</table>

Sensory Analysis

The control sample had a significantly lower score for sweet taste and higher score for butter flavor when compared to the treatment samples (Table 2). The control sample also had highest scores for oxidized and stale although not significant for all sample comparisons. Treatment 2 had the lowest scores for oxidized and stale although not significantly lower for all sample comparisons. The control sample had the lowest liking score (Table 3). When combining the two top categories, 66.9% consumers thought that treatment 2 was better than or equal to milk they normally drank as compared to 27.7% for the control (Table 4). Treatment 2 had the highest liking score although not significantly higher when compared to the other treatments.
Table 2. Mean scores\textsuperscript{1} for flavor attributes of 2 % UHT milk

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sweet</th>
<th>Sour</th>
<th>Salt</th>
<th>Bitter</th>
<th>Dairy</th>
<th>Cooked</th>
<th>Oxidized</th>
<th>Stale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.38\textsuperscript{2}</td>
<td>0.24</td>
<td>0.27</td>
<td>0.11</td>
<td>0.64</td>
<td>0.91</td>
<td>0.67</td>
<td>0.65</td>
</tr>
<tr>
<td>Treatment 1</td>
<td>0.9\textsuperscript{B3}</td>
<td>0.3\textsuperscript{A}</td>
<td>0.4\textsuperscript{A}</td>
<td>0.2\textsuperscript{A}</td>
<td>5.5\textsuperscript{A}</td>
<td>1.6\textsuperscript{A}</td>
<td>1.8\textsuperscript{A}</td>
<td>1.4\textsuperscript{A}</td>
</tr>
<tr>
<td>Treatment 2</td>
<td>1.6\textsuperscript{A}</td>
<td>0.3\textsuperscript{A}</td>
<td>0.3\textsuperscript{A}</td>
<td>0.1\textsuperscript{A}</td>
<td>6.0\textsuperscript{A}</td>
<td>1.9\textsuperscript{A}</td>
<td>1.3\textsuperscript{AB}</td>
<td>0.7\textsuperscript{AB}</td>
</tr>
<tr>
<td>Treatment 3</td>
<td>1.6\textsuperscript{A}</td>
<td>0.3\textsuperscript{A}</td>
<td>0.4\textsuperscript{A}</td>
<td>0.1\textsuperscript{A}</td>
<td>5.8\textsuperscript{A}</td>
<td>1.9\textsuperscript{A}</td>
<td>1.1\textsuperscript{B}</td>
<td>1.0\textsuperscript{AB}</td>
</tr>
<tr>
<td>Treatment 4</td>
<td>1.5\textsuperscript{A}</td>
<td>0.2\textsuperscript{A}</td>
<td>0.5\textsuperscript{A}</td>
<td>0.1\textsuperscript{A}</td>
<td>5.8\textsuperscript{A}</td>
<td>2.4\textsuperscript{A}</td>
<td>2.4\textsuperscript{A}</td>
<td>1.3\textsuperscript{AB}</td>
</tr>
</tbody>
</table>

\textsuperscript{1}\textit{N}=36 (2 replications x 9 panelists x 2 duplicates)
\textsuperscript{2}Minimum significant difference.
\textsuperscript{3}Means having the same letter are not significantly different (alpha=0.05).

Table 3. Mean overall liking scores\textsuperscript{1} of UHT milk

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Overall Liking\textsuperscript{3}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.69</td>
</tr>
<tr>
<td>Treatment 1</td>
<td>4.2\textsuperscript{B4}</td>
</tr>
<tr>
<td>Treatment 2</td>
<td>5.7\textsuperscript{A}</td>
</tr>
<tr>
<td>Treatment 3</td>
<td>6.1\textsuperscript{A}</td>
</tr>
<tr>
<td>Treatment 4</td>
<td>5.6\textsuperscript{A}</td>
</tr>
</tbody>
</table>

\textsuperscript{1}n=79 (40 persons evaluated rep1 samples; 39 persons evaluated rep 2 samples)
\textsuperscript{2}Minimum significant difference
\textsuperscript{3}A 9-point hedonic scale was used with 9 = like extremely, 8=like very much
7=like moderately, 6=like slightly, 5=neither like or dislike, 4=dislike slightly,
3=dislike moderately, 2=dislike very much, and 1=dislike extremely
\textsuperscript{4}Means having the same letter are not significantly different (alpha = 0.10)

Table 4. Summary of consumer's responses to the question comparing the milk sample to milk they normally drink

\%

39
In comparison to milk you normally Drink, the milk sample was:

<table>
<thead>
<tr>
<th>Question</th>
<th>Control</th>
<th>Trt 1</th>
<th>Trt 2</th>
<th>Trt 3</th>
<th>Trt 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Better than</td>
<td>13.9</td>
<td>13.9</td>
<td>15.1</td>
<td>13.9</td>
<td>13.9</td>
</tr>
<tr>
<td>Equal to</td>
<td>13.9</td>
<td>37.9</td>
<td>51.8</td>
<td>40.5</td>
<td>45.5</td>
</tr>
<tr>
<td>Worse than</td>
<td>72.1</td>
<td>48.1</td>
<td>32.9</td>
<td>45.5</td>
<td>40.5</td>
</tr>
</tbody>
</table>

The control sample had the lowest score for sweet taste and the highest score for oxidized flavor although not significant for all treatment comparisons (Table 5). The score for butter flavor for the control sample was significantly higher when compared to the treatment samples. The control sample had the lowest liking score (Table 6). Table 7 contains the summary of consumer's responses to the question comparing the milk samples to milk they normally drink. When combining the two top categories, 72.2% consumers thought that treatment 4 was better than or equal to milk they normally drank as compared to 20.3% for the control (Table 7). The percent of consumers who thought that treatments 1, 2, and 3 was better than or equal to milk they normally drank was 54.4%, 59.5%, and 58.3% respectively.

Table 5. Mean scores\(^1\) for flavor attributes of 2% UHT milk - Test

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Stale</th>
<th>Sweet</th>
<th>Sour</th>
<th>Salt</th>
<th>Bitter</th>
<th>Dairy</th>
<th>Cooked</th>
<th>Oxidized</th>
<th>Stock</th>
<th>Buttery</th>
<th>Sour</th>
<th>Salt</th>
<th>Bitter</th>
<th>Dairy</th>
<th>Cooked</th>
<th>Oxidized</th>
<th>Stock</th>
<th>Buttery</th>
<th>Sour</th>
<th>Salt</th>
<th>Bitter</th>
<th>Dairy</th>
<th>Cooked</th>
<th>Oxidized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.0(^A)</td>
<td>0.3(^A)</td>
<td>0.2(^A)</td>
<td>6.2(^A)</td>
<td>2.1(^A)</td>
<td>2.0(^A)</td>
<td>0.8(^A)</td>
<td>0.7(^A)</td>
<td>0.61</td>
<td>0.82</td>
<td>0.51</td>
<td>0.40</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment 1</td>
<td>1.6(^A)</td>
<td>0.4(^A)</td>
<td>0.4(^A)</td>
<td>0.1(^A)</td>
<td>6.0(^A)</td>
<td>2.4(^A)</td>
<td>1.4(^A)</td>
<td>0.4(^A)</td>
<td>0.62</td>
<td>0.7</td>
<td>0.55</td>
<td>0.40</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment 2</td>
<td>1.4(^A)</td>
<td>0.3(^A)</td>
<td>0.4(^A)</td>
<td>0.1(^A)</td>
<td>6.1(^A)</td>
<td>1.9(^A)</td>
<td>1.1(^B)</td>
<td>0.3(^B)</td>
<td>0.63</td>
<td>0.7</td>
<td>0.56</td>
<td>0.40</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment 3</td>
<td>1.2(^AB)</td>
<td>0.3(^A)</td>
<td>0.3(^A)</td>
<td>0.1(^A)</td>
<td>6.2(^A)</td>
<td>1.8(^A)</td>
<td>1.1(^B)</td>
<td>0.4(^A)</td>
<td>0.62</td>
<td>0.7</td>
<td>0.56</td>
<td>0.40</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment 4</td>
<td>1.3(^AB)</td>
<td>0.3(^A)</td>
<td>0.4(^A)</td>
<td>0.2(^A)</td>
<td>6.3(^A)</td>
<td>1.7(^A)</td>
<td>1.3(^A)</td>
<td>0.6(^A)</td>
<td>0.63</td>
<td>0.7</td>
<td>0.56</td>
<td>0.40</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)N=32 (2 replications x 8 panelists x 2 duplicates)
\(^2\)Minimum significant difference.
\(^3\)Means having the same letter are not significantly different (alpha=0.05).

Table 6. Mean overall liking scores\(^1\) of UHT milk

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Overall Liking(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>-------</td>
</tr>
<tr>
<td>Control</td>
<td>3.8 ^A</td>
</tr>
<tr>
<td>Treatment 1</td>
<td>5.9 ^A</td>
</tr>
<tr>
<td>Treatment 2</td>
<td>6.0 ^A</td>
</tr>
<tr>
<td>Treatment 3</td>
<td>6.0 ^A</td>
</tr>
<tr>
<td>Treatment 4</td>
<td>6.2 ^A</td>
</tr>
</tbody>
</table>

1 n = 79 (40 persons evaluated rep 1 samples; 39 persons evaluated rep 2 samples)
2 Minimum significant difference.
3 A 9-point hedonic scale was used with 9=like extremely, 8=like very much, 7=like moderately, 6=like slightly, 5=neither like nor dislike, 4=dislike slightly, 3=dislike moderately, 2=dislike very much, and 1=dislike extremely.

Means having the same letter are not significantly different (alpha=0.05)

Table 7. Consumer responses to the question: “In comparison to milk you normally drink, the milk sample was”

<table>
<thead>
<tr>
<th>Question</th>
<th>Control</th>
<th>Trt 1</th>
<th>Trt 2</th>
<th>Trt 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Better than</td>
<td>3.8%</td>
<td>13.9%</td>
<td>12.7%</td>
<td>16.5%</td>
</tr>
<tr>
<td>Equal to</td>
<td>57.0%</td>
<td>16.5%</td>
<td>40.5%</td>
<td>46.8%</td>
</tr>
<tr>
<td>Worse than</td>
<td>79.7%</td>
<td>45.6%</td>
<td>40.5%</td>
<td>41.8%</td>
</tr>
</tbody>
</table>
Gas Chromatography: Concentrations of key volatile compounds recovered from Electroheated and control were determined. Significant differences in the volatile compounds (2-pentanone, 2-hexanone, 2-heptanone, and dimethylsulphide) between the control and electroheated milk could not be determined. We are in the process of refining this technique for effective detection of these compounds.

Regardless of the treatment level combination, none of the electroheated samples showed identifiable differences in profiles of volatile compounds. Comparing the average electroheated data to the control sample, a total of thirteen volatile compounds were identified as having a potential sensory significance. The carbonyl compounds 2-heptanone, 2-nonanone, and nonanal were approximately 9-fold higher in the electroheated samples. Typically considered products of oxidation of unsaturated fatty acids, they are characterized as having green grass-like odors (Moio, et al, 1993). In contrast to the carbonyl compounds, the presence of two heat process-derived compounds, 3-furanmethanol and tetrahydro-2-furanmethanol, were identified only in the control samples. These compounds are associated with the development of heated flavors in products such as baked breads and caramel and are reported to have a roasted aroma. No significant differences were found between the concentrations of either phenolic or acidic compounds, however, these compounds are potent aroma-imparting compounds and may play some role in the overall background flavor of the milks. Finally, the control sample had a significantly higher amount of total esters, namely butyl acetate, than the electroheated milks.

Summary: Protein denaturation and sensory evaluation using trained and consumer panel indicated that the flavor of electroheated milk was better than the commercial variety. Experiments using GC indicated that there were no detectable differences between any of the electroheated samples and the only viable comparisons were with the control. However, when compared with a typical UHT product (eg. Gossners) the electroheated milk was very low in volatiles. Additional trials will be conducted to correlate this with the volatile compound analysis.

References


This information is important for meeting the contractual agreement under which your research was funded.

**Invention Disclosures: (Title, Date)**

___none___

**Patents: (Title, Date, #)**

___none___

**Licensing Activities:**

___none___

**Discoveries:**

___none___
Western Dairy Center
Project Report
Reporting Period January 1, 1997 — December 31, 1999

Principal Investigators: Marie K. Walsh, Assistant Professor, Department of Nutrition and Food Sciences, Utah State University. Paul A. Savello, Associate Professor, Department of Nutrition and Food Sciences, Utah State University.

Co-Investigators:

Project Title: Whitening of skim milk using a continuous-flow, immobilized enzyme reactor.

Institution’s Project #: 97084

Project Completion Date: 6-30-00

Research thermal and mechanical processing methods to improve sensory and color aspects of whiteness, texture and flavor.

Modifications to Project/Budget:
None

Project Objectives: (Include any revisions to objectives)
To develop a suitably active immobilized protease for use in skim milk whitening. To characterize the immobilized enzyme catalyst for operational stability and productivity in a fluidized-bed reactor with respect to skim milk whitening.

Project Summary: (Suitable for inclusion in Center documents released to the public)
Limited proteolysis with milk clotting enzymes can increase the whiteness of skim milk. The proteases porcine pepsin and chymosin were immobilized onto nonporous ceramic, glass, and controlled pore glass (CPG) beads. The enzymes were coupled to beads either directly or via crosslinker proteins. Pepsin, immobilized onto CPG beads via crosslinker proteins exhibited the best properties with respect to enzymatic activity, stability, and whitening efficiency. The Hunter L value (whiteness) of this immobilized enzyme treated skim milk was 79.5, which approached the whiteness of 1% fat milk. Immobilized proteases whitened skim milk more effectively than soluble proteases. Most recently we have determined the L value of skim milk at various temperatures from 4 to 90C. The L value increased with increasing temperature throughout the range tested. For samples not heated above 50 C, these increases were completely reversible on cooling. Partial reversibility was observed with samples between 50 and 90C. Milk
whiteness was also determined at different pH values ranging from 5.0 to 8.2 at temperatures of 4, 20, and 30C. The L value increased with decreasing pH and increasing temperature. The maximum L value was obtained at pH 5.0 and 30C. The temperature-dependent dissociation of major caseins was investigated by size exclusion chromatography. Free soluble kappa-casein and beta-casein were found at 10C.

1. Significant Progress against Objectives:
We have developed a suitable active immobilized enzyme reactor for the whitening of skim milk. The immobilization support, methodology, and milk clotting enzyme used significantly influenced the whiteness of skim milk.

Enzyme Immobilization
Three types of matrices were used, non-porous ceramic and glass beads, and controlled-pore glass (CPG) beads. Acid-cleaned beads (2 g) were silanized with 3-aminopropyltriethoxysilane according to Walsh and Swaisgood (1993). Aminopropyl beads were activated using 2% (v/v) glutaraldehyde in 50 mM sodium phosphate buffer, pH 6.5, and allowed to react at room temperature for 1 h on an orbital shaker. Excess reagent was washed off with distilled water. Pepsin or chymosin was coupled to activated beads either directly or via crosslinker proteins, WPI.

For direct immobilization, the enzyme solution (2-3 mg protein/ml) was added to beads and allowed to react at room temperature for 4 hr on an orbital shaker. Beads were thoroughly washed with simulated milk ultrafiltrate (SMUF) (Jenness and Koops, 1962) to remove all traces of free enzyme. For immobilization involving a crosslinker, a solution of 750 mg WPI in 25 ml 50 mM sodium phosphate buffer, pH 6.5, was mixed with the activated beads for 1 hr at room temperature. Excess protein was removed with distilled water and the WPI-coated beads were reduced by the addition of 100 mg of sodium borohydride (Cheryan et al. 1976). The reduced, WPI-coated beads were activated with 2% glutaraldehyde as described above. After completely washing off excess glutaraldehyde, enzyme solution (2-3 mg protein/mL) was added and allowed to react at room temperature for 4 h. Sodium borohydride (100 mg) was added slowly to the enzyme-coupling beads before thoroughly washing with SMUF buffer to remove free enzyme.

Whitening skim milk with immobilized proteases
The immobilized enzyme preparations, or biocatalyst, were housed in 2.5 x 10 cm glass columns. Approximately 25 ml pasteurized skim milk was circulated through the reactors via a peristaltic pump at room temperature. Sodium azide (0.02%) was added to the milk to prevent bacterial growth. Flow rates were measured by timing the volume of effluent collected in a graduated cylinder. Before the start of the experiment, the biocatalyst was washed with 25 ml skim milk which was discarded. During the experiments, 15 ml skim milk was collected at appropriate intervals for color determination, then returned to the column after recording color measurement.

For whitening skim milk with soluble enzymes, different amounts of enzymes (10
to 500 micro g) were added to skim milk and the change in L value over time was determined. The concentration of protein in each enzyme preparation was measured using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA).

Enzyme Activity and Color Measurements

Immobilized and soluble enzyme activities were determined using a fluorometric method (Molecular Probes, Inc. Eugene, OR). Rennin substrate (Molecular Probes) stock solution (1 g substrate dissolved in 877 micro L dimethyl sulfoxide), 12 microL., was added to protease-immobilized glass beads in 0.05 M sodium phosphate buffer (pH 6.0) in a cuvette. Activity was measured by monitoring the increase in fluorescence signal at 490 nm for 7-8 min and reported as delta RFU/min/g beads. The concentration of active enzyme immobilized onto the solid support was determined based on soluble enzyme standards.

To monitor leaching of enzyme from the matrix, the enzyme treated milk sample was collected and acidified with glacial acetic acid to coagulate the casein fraction. Whey, 0.5 ml, was collected from the sample and adjusted to pH 6.0 with 1 N NaOH before determining the protease activity as described above. Whey from untreated skim milk was used as a control.

Immobilized or soluble enzyme treated skim milk (20 ml) was collected and L values (milk whiteness) were measured at appropriate intervals using Hunter D25D2A color meter (Reston, VI).

Heat Treatment

Samples of skim milk (20 ml) were equilibrated in a water bath (antifreeze added to water when cooled to 10C or below) to a range of temperatures (4, 10, 20, 30, 40, 50, 60, 70, 80, and 90C) for 30 min. Samples were quickly removed from the bath, and the L value measured. The same samples were then cooled to 4C for 24 h, and the L value determined again.

Size Exclusion Chromatography

Macroosphere size exclusion column was obtained from Alltech Associates, Inc. (Deerfield, IL). The Column dimension was 300 x 7.5 mm. The column was packed with controlled pore spherical silica (1000Å). In order to separate the casein and serum fractions, two columns were joined in series. Skim milk samples (100 microl) were chromatographed with simulated milk ultrafiltrate (SMUF) (Jenness and Koops, 1962) at 10, 15, 20C (maintained by water jacket, Alltech Associates, IL) and at 30, 40C (maintained by column heater, Bio-Rad, CA) at a flow rate of 0.5 ml / min.

Gel Electrophoresis

Gel electrophoresis was carried out on polyacrylamide gels by the method described by Laemmli (1970) with 15% acrylamide. Gels were stained with Coomassie Brilliant Blue and destained with methanol/acetic acid solution. Gels were analyzed on a densitometer (Alpha Innotech Imager).
pH Adjustment

pH values were adjusted by 2M HCl or 2M NaOH into 30 ml skim milk with an ORION 520A pH-meter (Boston, MA). Skim milk color then was measured at each pH value at temperatures of 4, 20 and 30C using Hunter colorimeter.

2. Significant Conclusions:
Limited proteolysis with milk clotting enzymes increased the whiteness of skim milk which may result from an increase in the micelle size. The proteases porcine pepsin and chymosin were immobilized onto nonporous ceramic, glass, and controlled pore glass (CPG) beads. Among the three support materials, only the CPG beads displayed sufficient enzymatic activity to warrant continued studies. The enzymes were coupled to beads either directly or via crosslinker proteins. Pepsin, immobilized onto CPG beads via crosslinker proteins, revealed the best properties with respect to enzymatic activity, stability, and whitening efficiency. The Hunter L value (whiteness) of this immobilized enzyme treated skim milk was 79.5, which approached the whiteness of 1% fat milk. Immobilized proteases whitened skim milk more effectively than soluble proteases.

Whiteness of skim milk was determined at various temperatures from 4 to 90C. The L value increased with increasing temperature throughout the range tested. For samples not heated above 50C, these increases were completely reversible on cooling. From 4 to 20C, milk whiteness could influenced by the amount of kappa-casein in casein micelle. From 20 to 50C, color change may be due to the swelling of micelle upon heating. Partial reversibility was observed with sample between 50 and 70C the increasing whiteness in this range is possibly caused by the denaturation of whey proteins. Above 70C, the change in L value was not reversible upon cooling. This may involve the release of soluble casein and subsequent attachment to the micelles.

Milk whiteness was also determined at different pH values ranging from 5.0 to 8.2 at temperatures of 4, 20, and 30C. The L value increased with decreasing pH and increasing temperature. Maximum L value of 80.0 was obtained at pH 5.0 and 30C which is higher than the L value of 1% fat milk of natural pH at room temperature. Decreasing the pH of skim milk dissolves the calcium colloidal phosphate and reduces the binding of Ca to casein micelle which could result in a loosening of micelle and subsequent swelling of micelles. This may lead to higher L values.

The temperature-dependent dissociation of major caseins was investigated by size exclusion chromatography. Free soluble kappa-casein and beta-casein were found only at 10C. At higher temperatures, soluble beta-casein reassociates with the micellar structure due to the reinforced hydrophobic interaction.

In summary, limited proteolysis with soluble or immobilized milk clotting enzymes, heat treatment and pH influence the whiteness of skim milk at 25C. The usefulness of these treatments at refrigeration (4C) temperatures has yet to be determined.
3. Anticipated Problems/Delays:
None, this project is completed

Publications:
XIAOSHAN LI and M/RIE K. WALSH. INFLUENCE OF LIMITED PROTEOLYSIS WITH IMMOBILIZED OR SOLUBLE ENZYMES ON THE WHITENESS OF SKIM MILK in press for Journal of Food Biochemistry

Theses:
INFLUENCE OF LIMITED PROTEOLYSIS, HEAT TREATMENT AND pH ON THE WHITENESS OF SKIM MILK, X. Li

Published Abstract:
Immobilized pepsin for skim milk whitening. X. Li and M.K Walsh. 1999 Annual IFT Meeting. Chicago,IL.

Presentations:
None

Patent/Invention Disclosures:

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Visitors Hosted:
None
Introduction

Free ionic iron is a potent catalyst for lipid oxidation. Ionic iron is released from the heme group of meat pigments during cooking. The ionic iron contributes to oxidized, or warmed over flavor (WOF), especially in precooked meats that are reheated before serving. Ionic iron is hypothesized to bind to phospholipids, where it facilitates electron transfer reactions, ultimately leading to lipid degradation. I proposed that milk phosphoproteins may bind ionic iron, thus inhibiting lipid oxidation. I also proposed that calcium in dried milk mineral fractions may inhibit oxidation by displacing iron from phospholipid binding sites.

Results

1. Acid casein, the major phosphoprotein in milk, did not inhibit lipid oxidation in an egg yolk model system, nor in cooked ground beef, pork, or turkey.
2. A. Dried milk mineral (MM) was a very effective antioxidant in all meat products tested, as measured by TBA values and sensory panel evaluation. At 2% MM, T3A values of cooked beef burger were < 0.4 after 14 days refrigerated storage, while control burger had TBA # > 1 after 1 day storage, and TBA # > 2 after 14 days. MM at 1% was sufficient to keep TBA # < 0.4 for 14 days storage of pork or turkey burger.

B. Cooked beef, pork, and turkey burger and meat balls with MM had no detectable rancid odor or flavor after 1 month frozen storage. After 2 months, controls had very intense rancid flavor. Beef and pork samples with MM had only slight rancid flavor, and rancid flavor was not detectable in cooked turkey. Thus, MM very effectively inhibited oxidation in cooked burger and meat balls during frozen storage.

C. Dried MM was about 25% calcium, 36% phosphate, and 18% citrate. In tests of cooked beef with each compound, phosphates were the most effective antioxidants, although calcium chloride and sodium citrate had some antioxidant activity. Samples with tripolyphosphate had TBA # < 0.5, compared to TBA # 0.8 for cooked beef samples with sodium monophosphate, after 14 days refrigerated storage. A combination of calcium and tripolyphosphate was a highly effective antioxidant system, with TBA # 0.25 for cooked beef at 14 days refrigerated storage.

D. The method of addition of MM was compared (dry or with 10% added water, based on meat weight). Adding MM with water was slightly more effective, although samples made by both methods had TBA # < 0.4 after 14 days refrigerated storage of cooked beef samples. From a practical standpoint, it is desirable to add the MM with the meat during mixing and grinding, without added water. White MM particles were visible before, but not after cooking.

1. Significant Progress against Objectives:

Project is completed. This is the final report.
2. **Significant Conclusions:**

Conclusions

Although casein had no antioxidant activity, dried milk mineral (MM) was a highly effective antioxidant when added at a level of 2% to ground beef, or 1% to ground pork or turkey. At a cost of $3.0/lb MM and a use level of 2%, MM would cost $0.066/lb meat. Thus, cost is reasonable relative to the benefits of extended frozen shelflife. MM is also high in calcium, a mineral found in very low concentration in meat products. MM probably acts to bind ionic iron to multiple phosphate groups in the MM particles, thus blocking the catalytic effects of iron on lipid oxidation.

3. **Anticipated Problems/Delays:**

Project is completed.

**Publications:**

**Theses:**

**Published Abstract:**


**Presentations:**

**Patent/Invention Disclosures:**

6/30/99 to USU Foundation: Mr. Russ Price. Titled: Antioxidant properties of dried whey mineral fraction.

**Technology Transfer Activities**

For information on licensing contact:

**Visitors Hosted:**

None
Western Dairy Center
Project Report
Reporting Period January 1, 1998—December 31, 1999

Principal Investigators: Marie K. Walsh, Assistant Professor, Department of Nutrition and Food Sciences, Utah State University.

Co-Investigators: __________________________

Project Title: Developing an affinity purification procedures to purify prosaposin from whey

Institution’s Project#: 98094

Project Completion Date: 6-30-00

Increase the use of whey and whey products for health and nutrition applications/ positionings (include carries for nutraceuticals, pharmaceuticals, vitamins, essential nutrients).

Modifications to Project/Budget:
None

Project Objectives: Include any revisions to objectives)
1. To develop a bioselective adsorption matrix for affinity purification of prosaposin from whey. Factors to be considered include the type of immobilized ganglioside (i.e. GM1, GQ1b, and/or GT1b) and support (glass or polystyrene). 2. To determine the affinity of the interactions and dissociation requirements between immobilized gangliosides and prosaposin by analytical affinity chromatography. 3. To characterize bovine prosaposin with respect to enzyme activation and carbohydrate content.

Project Summary: (suitable for inclusion in Center documents released to the public)
In addition to the partial purification of prosaposin, we have purified both lactoferrin and transferrin using immobilized bovine gangliosides. Lactoferrin was purified from WPC and WPI using sodium phosphate buffer (pH 7) followed by sodium acetate buffer (pH 4) before elution of lactoferrin with 1 M NaCl in sodium acetate buffer. Lactoferrin was identified by N-terminal protein sequencing. From the intensities of the protein bands in SDS-PAGE, lactoferrin constitutes a minimum of 40% of the total protein in the salt eluted sample. Pretreated 1%WPI, heat treated and ultrafiltered with a 50 kDa membrane, showed the highest lactoferrin purity among protein sources, while WPI (10% wt/vol) showed the highest recovery. Bovine transferrin (BTF) was fractionated from bovine whey using ganglioside affinity chromatography. After loading the immobilized matrix...
with a 12% whey solution, the matrix was washed with sodium acetate buffer at pH 4 containing 1 M NaCl before elution of BTF with sodium phosphate buffers at pH 7. The ganglioside column showed a 74.2% BTF recovery from whey. In conclusion, immobilized gangliosides can be used to purify BLF and BTF from bovine whey. This novel matrix is stable to organic solvents, acidic solutions and drying.

1. Significant Progress against Objectives:

Ganglioside Purification

Gangliosides were purified from fresh bovine buttermilk (30% solids, Breaden Butter, Logan, UT). Buttermilk was diluted 1:10 with water and ultrafiltrated using a prep-scale-1TFF 2.5 ft² cartridge membrane with 10 kDa (Millipore, Bedford, MA) to remove lactose. The efficiency of lactose removal was confirmed with lactase enzymatic bioanalysis kit (Boehringer Mannheim, Indianapolis, IN). Moisture, protein, minerals and lipid content of lactose-free buttermilk were determined by oven, Kjeldahl, ash and Majonni, respectively. Lactose-free buttermilk was freeze-dried, and gangliosides were extracted using 20 vol. of organic mixture of chloroform:methanol:water (40:80:30 vol.) per gram dry solids (6). The sample was centrifuged at 11,000 x g and the supernatant was collected and evaporated under nitrogen gas. The total amount of gangliosides purified was determined by the periodate-resorcinol method using sialic acid as standard according to Jouardian et al. (9).

Ganglioside Immobilization

Controlled pore glass (CPG) beads (2000 Å, 120-200 mesh) Sigma, St. Louis, MO) were derivatized with 3-aminopropyltriethoxy silane and succinylated with succinic anhydride according to Walsh and Swaisgood (25). Acetic anhydride (10% by vol.) in acetone was used to cap excess amino groups. Gangliosides were saponified to produce the lysso-derivative containing a free amino group by the refluxing method (22). The OPA nmethod (O-phthalaldehyde) as described by Weimer and Berg (26) was used to confirm saponification of gangliosides. The carboxyl matrix was reacted with Sulfo-NHS (Pierce Chemical Co., Rockford, IL) in 0.01 M MES (pH 6) containing water-soluble carbodiimide (EDC). The lyso-gangliosides, dissolved in MES buffer (pH 6), were circulated through the Sulfo-NHS matrix to couple via amide bond formation for 12 hours at 4 °C using a peristaltic pump. Hydroxylamine (10 mM) was added to quench the reaction by hydrolyzing any unreacted NHS present. The efficiency of gangliosides immobilized was determined by measuring the amount of sialic acid on the matrix and the soluble gangliosides in the immobilization solution as described above.

Ganglioside Affinity Chromatography

Immobilized gangliosides (2 g beads) were packed into stop-flow columns (Pierce, Rockford, IL) and equilibrated with 0.05 M sodium acetate buffer (pH 4). For lactoferrin purification, crude and pretreated whey samples were applied to the column and circulated for 5 minutes at room temperature. Proteins were eluted by batch mode using four kinds of 0.05 M buffers in order: A= sodium phosphate, pH 7; B= sodium acetate, pH 4; C= sodium acetate, pH 4 containing 0.5 M NaCl; and D= sodium acetate, pH 4 containing 1.0 M NaCl.

For transferrin purification, whey sample (2% wt/vol) was applied to column and circulated for 5 minutes at room temperature. Bovine transferrin was purified by batch mode using four kinds of 0.05 M buffers; A = sodium acetate (pH 4), 1 M NaCl; B = sodium acetate (pH 4); C = sodium phosphate (pH 7); D = sodium phosphate (pH 7).
M NaCl. For the complete removal of lactoferrin and bovine serum albumin (BSA), the eluted whey sample was applied successively to the ganglioside column and followed by washing with sodium acetate buffer at pH 4 (buffers A and B) 4 times. BTF was eluted with sodium phosphate buffer at pH 7 (buffers C and D) and analyzed by SDS-PAGE under nonreducing conditions.

**Protein Concentration**
The protein concentration of samples eluting from the ganglioside column was determined by Bradford assay (Bio-Rad, Hercules, CA) with BSA as the standard.

**Results**
Bovine lactoferrin (BLF) and bovine transferrin (BTF) are major-iron transport and regulatory proteins found in bovine whey. BLF and BTF must interact with the eukaryotic cell surface to mediate their biological function of iron delivery and cellular functions of inflammatory and immunological reactions. As common components of eukaryotic cell surface, gangliosides were used for affinity purification of BLF and BTF. Bovine gangliosides were isolated from fresh buttermilk using a combination of ultrafiltration and organic extraction. Isolated gangliosides were covalently immobilized onto controlled-pore glass beads. The immobilized matrix contained 66 micrograms of gangliosides per gram beads.

The gangliosides affinity technique is a unique and novel method to purify BLF and BTF. In addition to BLF and BTF, immobilized ganglioside can be used to purify other proteins which interact with eukaryotic cell walls. This ganglioside affinity method presents several benefits in comparison with the methods used previously. This affinity chromatography procedure provides a rapid and efficient isolation of BLF and BTF without the need to pretreat whey. The inclusion of pH difference and sodium chloride in the chromatography buffers remove some of the nonspecifically absorbed whey proteins before elution of BLF or BTF. Gangliosides coupled to CPG beads have good mechanical properties and are stable to organic solvents, acidic solutions, and drying. The immobilized ganglioside column has been used repeatedly without apparent decrease in BLF binding capacity for 6 to 12 months. The support can be cleaned in ethanol, urea, or high salt buffers.

Bovine lactoferrin (BLF) was purified by affinity chromatography using immobilized gangliosides. After loading the matrix with whey protein (WPI or WPC), the matrix was washed with sodium phosphate buffer (pH 7) followed by sodium acetate (pH 4) before elution of lactoferrin with 1 M NaCl in sodium acetate buffer. Lactoferrin was identified by N-terminal protein sequencing. From the intensities of the protein bands in SDS-PAGE, lactoferrin constitutes a minimum of 40% of the total protein in the salt eluted sample. Pretreated whey isolate (1% wt/vol) showed the highest BLF purity among protein sources, while whey protein isolate (10% wt/vol) showed the highest recovery of BLF.

Bovine transferrin (BTF) was fractionated from bovine whey using ganglioside affinity chromatography. After loading the immobilized matrix with a 2% whey solution, the matrix was washed with sodium acetate buffer at pH 4 containing 1 M NaCl before elution of BTF with sodium phosphate buffers at pH 7. Con-A affinity and Mono-Q anion exchange chromatography were used for further purification. The ganglioside column showed a 74.2% BTF recovery from whey and BTF was enriched to 61% purity with Mono-Q chromatography. Bovine transferrin was identified by SDS-PAGE analysis, Western analysis, and isoelectricfocusing.

Bovine lactoferrin and bovine transferrin are of commercial interest because of their medical and nutritional benefits. Therefore, BLF and BTF purified using our affinity column could be applied for infant formulas to strengthen the immune system, specialty
dietary formulations to assist iron absorption, pharmaceuticals to treat harmful bacteria, and personal health items such as antibacterial toothpaste or anti-microbial cosmetics.

In conclusion, immobilized gangliosides can be used to purify BLF and BTF from bovine whey. These affinity purification procedures are suitable to obtain BLF and BTF from whey.

3. Anticipated Problems/Delays:
None

Publications:


Theses:
Affinity Purification of bovine lactoferrin and bovine transferrin using immobilized gangliosides. S.H. Nam. USU.

Published Abstract:

Presentations:
Institute of Food Technologists Annual Meeting, Chicago, IL.

Patent/Invention Disclosures:

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<td>For information on licensing contact: Provisional patent submitted</td>
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Visitors Hosted:
Scott Bloomer, Land O'Lakes, Inc.
Western Dairy Center
Project Report
Reporting Period January 1, 1998 — December 31, 1999

Principal Investigator: Marie K. Walsh, Assistant Professor, Department of Nutrition and Food Sciences, Utah State University.

Co-Investigators: 

Project Title: Developing an extruded whey protein meat extender suitable for use in coarse-ground meat products

Institution’s Project #: 98095

Project Completion Date: 12/31/00

Develop new applications for whey, NDM and their components.

Modifications to Project/Budget: None

Project Objectives: (Include any revisions to objectives)
We believe that therroplastic extrusion can be employed to produce a textured whey protein (TWP) having a stable, meat-like texture for use as an extender of coarse-ground meat products.

Objective 1. We will identify the physiochemical parameters of extrusion necessary to produce a TWP. The goal is to produce a TWP having a meat-like texture that is stable during cooking and consumption.

Step 1. We will employ response surface methodology to identify the physiochemical conditions that lead to the most extensive and stable protein cross linking during extrusion. The physiochemical factors to be considered include the temperature, pH, calcium concentration, moisture level, and extent of proteolysis of the whey protein.

Step 2. We will identify which of three likely polysaccharides (maltodextrin, carboxymethyl cellulose, and corn starch) imparts the most meat-like texture to the TWP when incorporated at different levels (10, 20, 30, and 40 wt% of the whey protein).

Objective 2. We will produce a TWP using the optimal extrusion conditions and formulation as identified in objective 1, and evaluate its...
use in meat patties and in crumbled meat. The patties and taco meat will be produced using ground beef mixtures formulated to include an all beef control, and 10, 20, and 30% of beef replaced with the TWP, and 30% of the beef replaced with a textured soy concentrate. The products will undergo sensory evaluation of texture, flavor, and juiciness by a consumer panel. Cook yield will be determined for the products and cohesiveness of patties will be instrumentally measured.

Project Summary: (Suitable for inclusion in Center documents released to the public)
Texturized vegetable proteins (TVP) are used extensively in institutional ground meat products as meat extenders. TVP is commonly made by extruding soy protein. Similar processing of whey protein would open new markets for whey protein. There are several that contribute to feasibility of texturizing whey protein via extrusion cooking, these include the type of added starch and extrusion temperature. We have shown that whey proteins can be texturized by thermoplastic extrusion for use as meat extenders. The textured whey proteins (TWP) can be used to replace up to 40% of the weight of hamburger patties without affecting consumer acceptance of the product.

1. Significant Progress against Objectives:  
Materials and Methods
Textured whey proteins were made by extruding a dry mix of: 2/3 whey protein concentrate (AMPC 800, American Meat Packers Cooperation, Ames IA) and 1/3 cornstarch (purchased locally). The whey protein concentrate (WPC) contains 80% protein, 4.6% fat, 4.5% ash (0.294% calcium), 4.4% carbohydrate, and 4.2% moisture. Calcium chloride dihydrate was added to the dry mix before extrusion. The NaOH, and HCl were added through the water source during extrusion. Lean and fat ground beef was purchased from the USU meat lab and fat content was determined using the Babcock method. Textured Procon®, a textured soy protein concentrate (Central Soya, Fort Wayne, IN) was the control used in panel one.

Extrusion
Extrusion was conducted on an APV Baker MPF19 twin-screw extruder. In the MPF19, dry feed and fluid are added separately and the components are mixed in the barrel. Collected samples were dried at room temperature overnight.

Sample preparation
Samples for both sensory and instrumental analysis were prepared at the same time. Formulation was carefully calculated for each panel. Hydration of textured whey and soy proteins was accomplished by adding water to the dry granules at a 1.5:1 (g/g) ratio. Product was allowed to stand at room temperature for at least 10 minutes to ensure complete hydration. Extenders were then mixed with lean and fat ground beef to give a final meat mix composition for the panel.

Patties were pressed into a 10-cm diameter and 1.2 cm height mold, and weighed a 1/4 lb. each. They were cooked on 350°F grills for four minutes on each side or until an internal temperature of 180°C was reached. Samples were then salted (first panel only) and cut though the center in into four equal, triangular pieces. Samples were served hot to panelists.
Two separate panels were conducted to determine consumer acceptance of TWP/PPIP in hamburger patties. Representative TWP samples were chosen for the first panel based on water holding capacity. The highest scoring TWP from the first panel was then evaluated in a second panel at three usage levels, 30, 40, and 50%.

Panel 1
In the first panel, 83 participants tested six samples including: 1) TWP extruded with water, 2) TWP extruded with 1 M HCl, 3) TWP extruded with 0.2 M NaOH, 4) TWP extruded with water with 1.6% alum (w/w protein) added, 5) a textured soy protein extender, and 6) an all beef control. The objective was to compare the acceptability of TWP to commonly used products. All meat extenders were added to the ground beef at the 30% level (weight/combined/weight patty). All patties were standardized to 13.6% fat.

Panel 2
In panel two, three usage levels of the most acceptable TWP from panel 1, TWP/PPIP extruded with 0.2 M NaOH, were tested, 30, 40, and 50%, and compared to an all beef control. Salt was added to each sample before patties were formed at 2 1/2 t per ~ 5 1/2 lb batch. All patties were standardized to 13.6% fat. Samples were tested by 88 panelists.

Sensory panel evaluation
Variables were assigned random three-digit numbers and rotated in ballot position to prevent bias. Panelists served hot samples in booths, under red lights, in a simulated consumer panel. They were asked to evaluate samples on a hedonic scale from 1 (dislike extremely) to 9 (like extremely) with a median of 5 (neither like nor dislike). FP panelists scored each sample for tenderness, juiciness, texture, flavor, and overall acceptability.

Textural analysis
Instrumental analysis was conducted on ten patties from each variable for each panel. Measurements were made using the USU Penetrometer, which measured the peak force required to break the patties. Measurement of raw and cold cooked weight, height, and diameter were made for each patty. Patties were cooked with those used in sensory analysis, but cooled to room temperature before analysis.

Results
In taste panel one, TWP extruded with 0.2 M NaOH and all beef control samples had significantly higher scores (p < 0.05) than all other samples in tenderness, texture, flavor and overall acceptability. For juiciness, TWP extruded with water was not significantly different (p < 0.05) from the two previously mentioned. The TWP extruded with 0.2 M NaOH was the only sample with average scores above 6 (like slightly) in every category. Acid adjusted TWP had lowest scores for texture, and TSP had lowest scores in favor. All beef and TSP samples required higher (p < 0.05) peak forces than the TWP samples. Within TWP samples, 0.2 M NaOH had the highest to breakage peak force.

In taste panel two, no sensory differences (p < 0.05) were found between the all beef control and patties with 30% and 40% TWP added. However, 50% TWP samples received lower scores for texture, flavor, and overall acceptability. There were no differences among the peak forces of patties with TWP added, and all had lower peak forces than the all beef control. However, the differences due to peak force do not seem to affect the overall acceptability of the samples.

Conclusion
This research shows that whey protein extruded with base is a product that can be used in meat patties up to at least 40% level. This research shows high usage potential for...
textured whey proteins as meat extenders.

3. Anticipated Problems/Delays:
None

Publications:

Theses:

Published Abstract:


Presentations:
Same as above

Patent/Invention Disclosures:

Technology Transfer Activities
For information on licensing contact:
Provisional Patent filed.

Visitors/Hosted:
Western Dairy Center
Project Report
Reporting Period January 1, 1998 — December 31, 1999

Principal Investigators: Deborah R. Gustafson
Co-Investigators: 

Project Title: Appetite Suppressing Properties of a Peptide from Milk

Institution’s Project #: 89099

Project Completion Date: Summer 2000

To develop opportunities to increase the use of whey for health and nutrition applications

Modifications to Project Budget:

Project Objectives:
1. To determine the short-term effect of CMP on food intake in healthy men and women.
2. To identify changes in biomarkers of satiety as a function of CMP ingestion in healthy women. Different doses and timing strategies will be used.
Project Summary:
The purpose of this project is to investigate the effects of a milk protein (caseinomacropeptide, CMP) on the regulation of food intake in humans. It has been suggested that CMP suppresses appetite in humans. Also, the marketability of milk would be greatly improved. Milk, milk products, and other dietary formulations based on milk components, would empirically have a very large market in the United States and the world. National survey data indicate that approximately one-third or 58 million American adults 20 years of age or older are overweight; and the prevalence of overweight continues to increase. The health consequences of overweight and obesity include heart disease, diabetes, high blood pressure, and certain cancers. As a result, obesity is the second leading cause, after cigarette smoking, of preventable deaths in the United States.
The increasing trends in overweight, in addition to higher energy intakes, are occurring despite the growing prevalence of fat replacers and low-fat foods sold in American markets, and the interest of Americans in these products. Thus, alternative strategies must be developed to decrease weight and obesity among Americans. One way of accomplishing this goal is through the ingestion of naturally-occurring food substances that decrease food intake. CMP may be one of these substances.
The objectives of this project are to determine the short-term effect of CMP on appetite suppression in humans and to identify changes in blood markers that may be associated with satiety following CMP intake.

1. Significant Progress against Objectives:

2. Significant Conclusions for Project Objective #1:
Background: Milk proteins possess biological activity, such as regulation of food intake. Caseinomacropeptide (CMP) is a predominant breakdown product of casein in the human stomach. CMP may be involved in the regulation of food intake in humans.
Objective: The purpose of this project was to characterize the effects of CMP on satiety and satiation by measuring the absolute amount of food consumed at usual meal times and through subjective motivation to eat measures.
Design: The study design was a human feeding study involving 20 male and 32 female adults. The following beverage treatments were prepared: 0.4% CMP solution, 2.0% CMP solution, vehicle alone, and water containing a colorant and clouding agent. Subjects were randomized into the study using a Latin Square randomization. Treatment beverages and ad libitum lucies were consumed on four separate occasions at the Study Center. Beverages were consumed one hour prior to lunch. After lunch, subjects were encouraged to...
leave, but completed a 10 standardized questionnaire every hour throughout the afternoon and evening to assess feeling of hunger and stomach fullness. Subjects also kept track of all food and beverages consumed away from the Sucy Center during the four study days.

Results: Under these experimental conditions, CMP had no effect on energy intake or weight of food consumed at lunch or for the remainder of the day. CMP also had no effects on subjective indicators of satiety.

Conclusions: Intake of CMP before a midday meal had no effect on regulation of food intake over a short-term period.

3. Anticipated Problems/Delays:
There has been a delay initiating Objective #2, the biomarkers portion of the project, as we have waited for adequate information on other studies currently being conducted in CMP and appetite.

Publications:
In preparation:
"Appetite is not influenced by a unique milk peptide" Authors: DR Gustafson, DJ MacMahn, J Morrey, and R. Nan. To be submitted to the American Journal of Clinical Nutrition.

Theses:

Published Abstract/Presentation:
Western Dairy Cater
Project Report
Reporting Period July 1, 1997 — Dec 31, 1999

Principal Investigators: D. Jeffery R. Broadben, Ila State University
                          D. Jeffery R. Broadben, Ila State University
                          D. James L. Steele, University of Wisconsin-Madison
                          D: Mark E. Johnson, Center for Dairy Research and
                          Dr. Scott A. Rankin, University of Maryland

Project Title: Improvement of low-fat Cedar cheese through
              identification and characterization of microbial
              enzymes responsible for the conversion of aromatic
              amino acids into off flavor compounds in cheese.

Institution's Project #: 0787

Project Completion Date: 6-3-0

National Research Plan [1997: Priority: 1; Goal: 1; Acid: 2;]
Clarity: Which organisms are responsible for cheese flavor positive and negative.
Understand how these organisms assert their influence.

Modifications to Project/Budget

Project Objectives: (Include any revisions to objectives)
The overall objective of this proposal is to identify and characterize key enzymes
and mechanisms which are primarily responsible for the conversion of
aromatic amino acids into off flavor compounds in low-fat Cheddar cheese.

Objective 1. Investigate there of metabolic cross-feeding between starter,
adjunct, and nonstarter bacteria in the production or removal of
off flavor compounds.

Objective 2. Characterize the distribution of key enzymes to the conversion of
aromatic amino acids into flavor compounds.

Objective 3. Confirm the acids key enzymes in cheeses slurries and in low-fat
Cheddar cheese.

Project Summary: (Suitable conclusion in Center documents released to the public)
Development of off flavors is a significant problem in low-fat Cheddar cheese.
Compounds associated with green, medicinal or utensil, and floral or rosy off
flavors may arise via microbial metabolism of aromatic amino acids. Starter,
adjunct, and nonstarter lacticid bacteria may catabolize aromatic amino acids...
under conditions found in Cheddar cheese, and pathways involved in these reactions can facilitate the production of off flavor compounds. This project will investigate the contribution of metabolic cross-feeding between starter, adjunct, and nonstarter bacteria to the production or removal of aromatic off flavor compounds, the specific roles for selected enzymes in the production of these compounds, and confirm that these enzymes and pathways are functional in low-fat Cheddar cheese. Results from the project will facilitate industry efforts to understand and control flavor development in low-fat Cheddar cheese by providing new strategies, based on enzyme assays, gene probes, or recombinant DNA technology, that can be used to identify or develop starter systems which avoid or reduce development of utensil, medicinal, unclean, putrid, and floral off flavors in low-fat Cheddar cheese.

1. Significant Progress against Objectives:
Objective 1 is complete and experiments to address objectives 2 and 3 are well underway. Metabolic cross-feeding studies for Trp, Phe, and Tyr confirmed that these reactions are active under simulated cheese conditions (pH 5.2, 4% NaCl, no carbohydrate, 13-15°C). Those studies established that unlike the *Lactococcus lactis* (Lc. lactis) starter bacteria, *Lactobacillus casei* (Lb. casei) are able to metabolize p-OH-phenyl pyruvic acid into p-OH-phenyl lactic acid, a compound that is not associated with cheese off-flavors. As a result, overexpression of the enzyme that effects this conversion, D-2-hydroxyisocaproate dehydrogenase, should yield bacteria that can remove p-OH-phenyl pyruvic acid from the cheese matrix and thus help to prevent spontaneous degradation of p-OH-phenyl pyruvic acid into off flavor compounds. To test this hypothesis, we have cloned the *Lb. casei* gene encoding the NAD(H)-dependent D-2-hydroxyisocaproate dehydrogenase (D-HicDH) gene in the high copy number plasmid pTRKH2, and have successfully transformed this plasmid into *Lb. casei* and *Lactococcus lactis*. Work is now underway to determine how the increased dosage of this gene affects the ability of these two key bacteria to remove Phe, Tyr, and Trp aromatic intermediates from their medium.

Enzyme studies performed under objective 1 established that the conversion of Tyr, Phe, and Trp into the keto acid intermediates p-OH-phenyl pyruvic acid, phenyl pyruvic acid, and indole pyruvic acid, respectively, is primarily due to the action of the starter bacterium, *Lc. lactis*. Although our original objective was to inactivate the *Lb. casei* aromatic ATase gene, we now recognize that this approach would not be an effective strategy to reduce production of aromatic intermediates in cheese. In addition, recent work by our collaborator Dr. James Steele has shown that the lactococcal aromatic ATase is important to Met catabolism and therefore may be essential for the production of desirable cheese flavor compounds. As a result, we no longer plan to construct an ATase-deficient strain of *Lb. casei*. However, we have isolated a fragment of an *L. casei* aminotransferase (ATase) gene and are using it, in concert with other approaches, to clone the ATases from this bacterium so that we may study their contribution to cheese flavor chemistry. We also propose to determine whether production of aromatic (but not Met) intermediates can be redirected in *Lc. lactis* by overexpression of the D-HicDH gene. From these experiments we hope to
determine whether we can control aromatic amino acid catabolism and off-flavor production in cheese without sacrificing desirable Met catabolism.

2. Significant Conclusions:
Starer, adjunct, and nonstarter lactic acid bacteria are each able to catabolize aromatic amino acids in defined media under conditions found in Cheddar cheese, and pathways involved in these reactions can facilitate the production of off flavor compounds.

3. Anticipated Problems/Delays:
None.

Publications:

Theses:

Published Abstract:
none

Presentations:

Broadbent, J.R. Cheese curing and flavor development. Invited oral presentation for the 15th Cheese Making Short Course. February 9-11, Utah State University.


Patent/Invention Disclosures:
none

Technology Transfer Activities
For information on licensing contact:
Jeff Broadbent

Visitors Hosted:
none
Western Dairy Center
Project Report
Reporting Period July 1, 1997 — Dec. 31, 1999

Principal Investigator: Dr. Jeff Broadbent, Utah State University
Co-Investigators:
Dr. Mark Johnson, Wisconsin Dairy Center
Dr. James Steele, University of Wisconsin

Project Title: Growth of Non-starter Lactic Acid Bacteria in Reduced Fat Cheddar Cheese, Utah State University part.

Institution's Project #: 97085

Project Completion Date: 6-30-00

National Research Plan (1997): Priority: 1; Goal: 1; Tactic: 3;
Establish knowledge matrices relating cheese composition, flavor and the role of adjunct and nonstarter microorganisms.

Modifications to Project/Budget:
None

Project Objectives: (Include any revisions to objectives)
Objective 1 To establish the population dynamics between starter, non-starter, and adjunct bacteria during ripening of 50% reduced fat Cheddar cheese.

Objective 2 To establish the population dynamics between starter, non-starter, and adjunct bacteria during ripening of 50% reduced fat Colby cheese.

Objective 3 To construct derivatives of the adjunct Lactobacillus casei subsp. pseudoplanturn that are unable to co-metabolize citrate and lactate and to test the influence of the loss of this metabolism on the ability of the adjunct to grow in cheese.

Objective 4 To establish the impact on the sensory attributes of reduced fat Cheddar cheese to which adjunct bacteria have been added by monitoring the relationship between growth of starter, adjunct and non-starter bacteria and flavor attributes during aging of the cheese.

Project Summary: (Suitable for inclusion in Center documents released to the public)
Microbial studies of ripening cheese reveal that numbers of starter bacteria decline during maturation while those of, while those of nonstarter bacteria (NSLAB; in particular lactobacilli) increase to levels of $10^7-10^8$ CFU per gram.
of cheese. It is well established that starter, adjunct, and NSLAB can have a profound effect on the development of flavor in Cheddar cheese. The cause and effect relationship between these bacteria, however, has not been studied, nor is much known about mechanisms that enable these bacteria to maintain viability or proliferate in cheese. While the type and numbers of adjunct and starter bacteria can be controlled, the types of NSLAB still remain a matter of chance. It is the hypothesis of this project that certain adjunct bacteria can be used to control the NSLAB population to ensure proper flavor development. To test this hypothesis, we are investigating the effect of adjunct bacteria on the numbers and types of NSLAB in ripening cheese and the influence of cheese environment on NSLAB and adjunct populations.

1. Significant Progress against Objectives:
Work at Utah State University is focused on Objectives 1 and 2, while objectives 3 and 4 are being done at the University of Wisconsin-Madison. Our research on the population dynamics between starter, non-starter, and adjunct bacteria during cheese ripening has relied on random amplified polymorphic DNA (RAPD) fingerprinting of cheese isolates. Initial studies confirmed the utility of this technique for differentiation among individual strains of *Lactococcus lactis*, *Lactobacillus casei*, and *Lactobacillus helveticus*. For the cheese studies, 8 vats of 50% reduced-fat Cheddar and Colby cheese was manufactured at UW-Madison in November of 1998. Template DNA for RAPD was isolated from 80 individual colonies (10 per vat) collected on Rogosa or Ellikers agar after day 1, 2 weeks, 1 mo, 2 mo, 3 mo, 4 mo and 6 mo of ripening. RAPD fingerprints have now been collected from day 1, 2 mo, 4 mo, and 6 mo cheese isolates, and analysis of the 2 wk and 3 mo samples is underway. In addition, any cheese isolates which display a unique RAPD fingerprint are being identified by nucleotide sequence analysis of their 16S rRNA gene. By combining strain fingerprint and species data, we have been able to monitor the diversity of NSLAB in each of the cheeses over time and have been able to evaluate the ability of an adjunct *Lactobacillus* sp. strain to dominate the NSLAB biota. As part of this work, we also isolated DNA from our 6 mo-old experimental cheese and used it as a template for the amplification of bacterial 16S rRNA genes. It was our hope that this approach would allow us to speciate nonculturable bacteria that might dominate the NSLAB population. Unfortunately, the only species identified to date by DNA sequence analysis of 10 different 16S rDNA clones is the starter, *Lc. lactis*.

2. Significant Conclusions:
RAPD is an effective method for the analysis of NSLAB population dynamics in ripening cheese. Our data show that the NSLAB biota in cheese changes over time and that adjunct *Lactobacillus* sp. may not dominate the NSLAB biota throughout ripening.
Western Dairy Center
Project Report
Reporting Period July 1, 1997 — Dec. 31, 1999

Principal Investigators: Dr. Jeffery R. Broadbent, Utah State University
Co-Investigators: Dr. Charlotte Brennand, Utah State University
Dr. James L. Steele, University of Wisconsin-Madison
Dr. Mark E. Johnson, Center for Dairy Research

Project Title: Improvement of Cheddar cheese quality through identification and characterization of microbial enzymes responsible for the production or degradation of bitter peptides in cheese.

Institution’s Project: 97086

Project Completion Date: 6-30-00

National Research (1997): Priority: 1; Goal: 1; Tactic: 2;
Establish knowledge matrices relating flavor and role of starter, adjunct, and nonstarter bacteria to clarify which organisms are responsible for positive and negative attributes of cheese flavor and provide an understanding of how these organisms assert their influence.

Modifications to Project/Budget:
None

Project Objectives: (Include any revisions to objectives)
The overall objective of this proposal is to identify and characterize enzymes produced by starters and flavor adjunct bacteria which are responsible for the production or degradation of bitter peptides in Cheddar cheese.

Objective 1 Define the contribution of starter CEP specificity on peptide pools and bitterness in Cheddar cheese.

Objective 2 Develop a cheese-based test for bitterness in Cheddar cheese and establish persons that influence sensory perception of bitterness in Cheddar cheese.

Objective 3 Determine bitter taste thresholds for β-CN (f193-209) and αs1-CN (f1-9).

Objective 4 Define the contribution of Lacticillus helveticus CNRZ32 peptide to degradation of β-CN (f193-209) and αs1-CN (f1-9).

Objective 5 Construct L. lactis SK11 derivatives with enhanced activity of peptides demonstrated to be important in hydrolysis of β-CN (f193-209) & αs1-CN (f1-9).
Project Summary: (Suitable for inclusion in Center documents released to the public)

Bitterness is a significant problem in Cheddar cheese, and this defect is particularly common in low-fat cheeses. Bitterness has been a problem in cheese for decades, but modern consumer preference for mild-tasting Cheddar has lent greater significance to the impact of bitterness on consumer economics. Bitterness is caused by the accumulation of hydrophobic peptides produced by some starter bacteria and chymosin. Starter proteinase specificity is the primary determinant in whether or not a starter culture produces bitter peptides. Fortunately, bitter peptides produced by chymosin and starter bacteria can be degraded by intracellular peptidases from starters and direct bacteria, but the relative contribution of individual peptidases to these reactions remains unknown. This project is working to identify and characterize microbial enzymes responsible for the production or degradation of bitter peptides in cheese. Results from the study will facilitate industry efforts to understand and control flavor development in Cheddar cheese by providing new strategies to identify or develop starter systems which eliminate or control bitter flavor defect in full and low-fat Cheddar cheese.

1. Significant Progress against Objectives:

Previous research by our group has shown that teactococcal cell envelope proteinase (CEP) is a primary determinant in whether or not a starter culture will produce bitter peptides. One of the limitations in that study was that peptide accumulation data were confounded by variability in the degree of autolysis and intracellular peptidase activity among strains of *Lactococcus lactis*. To overcome this limitation, Dr. Broadbent’s laboratory constructed a series of isogenic strains which differ only in proteinase specificity and which lack the gene for the major lactococcal autolysin, AcmA. The proteinase which were evaluated included the *L. lactis* Wg2 group e proteinase, CEP, the *L. lactis* SK11 group a proteinase, and the group h proteinase from the bitter starter *L. lactis* S3. The proteinase specificity of each isogenic construct was confirmed by in vitro incubation of whole cells with α5-CN (f1-23) at pH 5.2 ± 45 NaCl and, for the S3 clone, by DNA sequence analysis of the substrate binding regions. Permission to perform sensory analysis on cheeses manufactured with these bacteria was then obtained from the Utah State Biosafety Committee and the JSU Institutional Review Board (for experiments with human subjects). 5% reduced-fat Cheddar cheese was manufactured at UW-Madison and HPLC analysis has confirmed that peptide accumulation in the experimental cheeses is occurring as predicted by the CEP specificity of each starter. Trained sensory analysis of the experimental cheeses after 2, 4, and 6 mo of ripening has established a clear role for CEP specificity in bitterness. As expected, strains carrying the group a, e, or h proteinase had low, intermediate, or high propensities for bitterness, respectively. These results confirm our previous finding that starter culture proteinase specificity is a key determinant of whether or not a cheese will develop bitterness.

A number of casein-derived peptides with bitter flavor notes have been characterized, but the actual peptides that are most frequently responsible for bitterness in cheese have not yet been identified.
to determine the contribution of specific peptides to bitterness in cheese have relied on sensory evaluation of peptides in aqueous solutions to measure bitterness. However, sensory studies have clearly established that taste thresholds for a compound increase when viscosity increases or when competing tastes are present. For this reason, the quantity of any peptide necessary to evoke a bitter response will always be much higher in cheese than in water, so water dispersion data cannot be reliably applied to cheese. Dr. Brennand's work has demonstrated that dispersal of bitter compounds in a cheese model system is a representative and effective means to study bitterness in cheese. To our knowledge, we are the first group to study the contribution of individual peptides to bitterness in model cheese systems, and our work on bitter taste thresholds for β-CN (f193-209) and α_s1-CN (f1-9) has provided valuable new insight into the role of specific peptides in bitterness. In the case of both peptides the bitter taste threshold was approximately 10-fold higher in the model cheese system than in water. When the bitter taste threshold of these peptides in the model cheese system were compared to the levels of these peptides observed in a bitter cheese, it was concluded that the α_s1-CN (f1-9) was primarily responsible for bitterness in this cheese. While the β-CN (f193-209) peptide likely had a complementary function, rather than a dominant role, in the perception of bitterness in this cheese.

The ability of lactic acid bacteria peptidases to hydrolyze bitter peptides to non-bitter peptides and amino acids is well established, but the relative contribution of individual enzymes to this process is largely unknown. The peptidase system of Lactobacillus helveticus CNRZ32, an adjunct that reduces bitterness in cheese, has been investigated in detail by Dr. Steele's laboratory. Genes for ten peptidases have been cloned and sequenced from this organism. Of these enzymes, the contribution of 2 general aminopeptidases (PepC and PepN), a proline-specific aminopeptidase (PepX), and two endopeptidases (PepO and PepE) to the hydrolysis of the known bitter peptides β-CN (f193-209) and α_s1-CN (f1-9) has been evaluated. Growth studies and studies with cell-free extracts (CFEs) of CNRZ32 and isogenic strains lacking one of the five peptidases mentioned above revealed that all of the mutants hydrolyzed these peptides completely to free amino acids. These results indicated that overlapping specificities in CNRZ32 peptidases were masking the effect of individual peptidases. To overcome this problem, we evaluated the rate of hydrolysis and the transition peptides formed by cell-free extracts of CNRZ32 and the five isogenic peptidase-deficient derivatives described above. Differences in the hydrolysis of β-CN (f193-209) were only observed between CNRZ32 and the mutant lacking PepN activity. These results indicated that PepC, PepX, PepO, and PepE have no detectable role in the hydrolysis of β-CN (f193-209) and that PepN initiates the N-terminal hydrolysis of this peptide. The observation that 50% of the transition peptides identified from β-CN (f193-209) had either a C-terminal Pro_204 or Pro_206 residue suggested that a post-proline endopeptidase was also involved in the hydrolysis of this peptide. Confirmation of a post-proline endopeptidase in CNRZ32 was obtained by the ability of CNRZ32 CFEs to hydrolyze C- and N-blocked β-CN (f203-209). The identification of a post-proline endopeptidase in CNRZ32 is significant, as this enzyme's substrate specificity suggests it may contribute to the hydrolysis of numerous bitter peptides. Hydrolysis of the α_s1-CN (f1-9) by CFEs from CNRZ32 and its isogenic
derivatives lacking one of the five peptidases previously described was evaluated. The primary peptide produced by all CFEs was αs1-CN (f1-7), suggesting either that an endopeptidase distinct from PepO and PepE or a carboxypeptidase was responsible for the formation of this peptide. Currently, the possible involvement of the post-proline endopeptidase in the formation of this peptide is under investigation.

2. Significant Conclusions:
Dr. Brennand's trained sensory panel has shown that although the concentration of β-CN (f193-209) in bitter S3 cheese is above the bitter taste threshold for water, it is well below the taste threshold for cheese. Thus, β-CN (f193-209) likely has a complementary function, rather than a dominant role, in the perception of bitterness in Cheddar cheese. In contrast, the bitter taste threshold for αs1-CN (f1-9) in cheese appears to lie very near the actual concentration of this peptide in bitter cheese. The latter finding supports our previous suggestion that αs1-CN (f1-9) may have a key role in bitterness in Cheddar cheese, and helps to illustrate the basis by which CEP specificity relates to a strain's propensity for bitterness. In addition, Dr. Steele's observation that cell-free extracts from Lactobacillus helveticus CNRZ 32 can degrade β-CN (f193-209) also supports our assertion that adjunct bacteria can be used to remove bitter peptides produced by the action of chymosin. Together, these observations indicate that we can develop starter systems which do not produce, but can still degrade, bitter peptides in Cheddar cheese.

3. Anticipated Problems/Delays:
none

Publications:

Theses:
none

Published Abstract:
none
Presentations:

Patent/Invention Disclosures:
none

Technology Transfer Activities
For information on licensing contact:
Jeff Broadbent

Visitors Hosted:
none
Western Dairy Center
Project Report
Reporting Period July 1, 1997 — December 31, 1999

Principal Investigators: Dr. Bart C. Weimer, Utah State University
Co-Investigators: 

Project Title: Microbial catabolism of Methionine to improve Cheddar cheese flavor - a comparative study of the relative contribution by starter cultures and flavor adjunct bacteria.

Institution’s Project #: 79088

Project Completion Date: 6-31-00

Develop/characterize/modify strains with particular flavor producing capabilities and enzymes linked to actual cheese flavor development.

Modifications to Project/Budget: None

Project Objectives: (Include any revisions to objectives)
Use bacteria with defined flavor producing capabilities as model systems for the study of enzymes and metabolites linked to the development of 1) desirable and 2) undesirable flavors in Cheddar cheese.

Project Summary: (Suitable for inclusion in Center documents released to the public)
Met metabolism in dairy related bacteria is under investigation. Brevibacteria produce more sulfur containing compounds during their metabolism than do lactic acid bacteria. Additionally, the mechanism by which production of methanethiol occurs is different than lactococci. The enzyme responsible for methanethiol production in brevibacteria was isolated to homogeneity and characterized – methionine gamma-lyase (MGL). Addition of MGL, whole cells of B. linens BL2 (commercially available from GB) with either GDL or L. cremoris S2 demonstrated that MGL and whole cells of BL2 produced significantly more volatile sulfur compounds (VSC) than S2 alone in slurries. L. cremoris S2 produced VSC at levels just above flavor threshold. Addition of MGL or BL2 produced 2 to 5 times more VSC than S2 alone. These increases were associated with the treatments and not contamination from other organisms. The predominant VSC were methanethiol, dimethyldisulfide, and dimethyltrisulfide. As the methanethiol content decreased the dimethyldisulfide content increased.
This observation suggested the redox potential was important, but was not measured.

Studies to screen other lactic acid bacteria (LAB) for Met utilization indicate LAB produced significantly less VSC than brevibacteria. Whole cells or cell free extracts produce VSC, but wholes cells produce less than cell free extracts, suggesting that Met transport may be important in the production rate.

Studies to isolate the MGL gene from brevibacteria are underway, but slow because the basic genetic information in brevibacteria is not known. Preliminary data are encouraging for locating, cloning, and sequencing the gene. PCR studies to amplify a fragment useful for locating the gene underway. Additional work is proceeding.

Additional questions related to the production of sulfur-containing amino acids in cheese are being evaluated. The role of this in relationship to the reduction of gene expression in LAB is being evaluated.

1. Significant Progress against Objectives:
All objectives are on time.

2. Significant Conclusions:
Brevibacteria produce more sulfur compounds from Met than lactic acid bacteria. While a broad range of MTPC occurs in lactococci and lactobacilli, it seems the mechanism of production varies between the two genera. MGL is active in slurries, suggesting that it maybe active in Cheddar cheese curd. Lactococci produce more VSC that expected in slurries, suggesting an unknown mechanism is at play.

The genes associated with MGL are being investigated now. The genetics of brevibacteria are significantly more difficult than those in other bacteria associated with cheese.

3. Anticipated Problems/Delays:
The student associated with this project quit to stay home with a new baby. I am in the process of finding a new student or post doc to finish the project.

Publications:


Theses:
Ben Dias – Ph.D. (completed spring ’99)
Kim Seefeldt – Ph.D. terminated due to student quitting

Published Abstract:

Presentations:
Weimer, B. C., 1999. Flavor compounds – bacteria and cheese. Land O'Lakes, Minneapolis, MN.


Weimer, B. C., 1999. Sulfur metabolism in dairy related bacteria. Institute of Food Technologists annual meeting, Biotechnology Section, Chicago, IL.


Patent/Invention Disclosures:

Technology Transfer Activities
For information on licensing contact:
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Visitors Hosted:
Paul Chiak – IFF
Chakra Wijesundera – Food Science Australia
Western Dairy Center
Project Report
Reporting Period July 1, 1999 — December 31, 1999

Principal Investigators: Bart Weimer, Utah State University
Co-Investigators:

Project Title: Use of non-lactic acid bacterial proteolytic enzymes to reduce bitter peptides in dairy products.

Institution’s Project #: 99211

Project Completion Date: 6/30/00

National Research Plan (1997): Priority: 4; Goal: 4; Tactic: 1

Modifications to Project/Budget:
None

Project Objectives: (Include any revisions to objectives)

Hypothesis:
Proteolytic enzymes associated with non-lactic acid bacteria will increase proteolysis that subsequently improves flavor development and reduces bitter peptides during cheese aging.

Objectives:
1) Determine the ability of 3 previously isolated proteases to degrade αs1-casein (f1-9) in Jenness-Koops buffer.
2) Determine the ability of 3 previously isolated proteases to degrade B-casein (f193-209) in Jenness-Koops buffer.
3) Determine the ability of these proteases to degrade αs1-casein (f1-9) and B-casein (f193-209) in direct acid cheese slurries.

Project Summary: (Suitable for inclusion in Center documents released to the public)

1. Significant progress against objectives:

2. Significant Conclusions:
Western Dairy Center
Project Report
Reporting Period July 1, 1997 — December 31, 1999

Principal Investigators: Dr. Bart C. Weimer, Utah State University
Co-Investigators: 

Project Title: Conversion of amino acids to short and branched-chain-fatty acids by starter and adjunct bacteria.

Institution’s Project #: 97089

Project Completion Date: 6-31-00

Understand how cheese matrix composition influences survival and metabolism of starter and adjunct cultures.

Modifications to Project/Budget:
None

Project Objectives: (Include any revisions to objectives)
To use bacteria with defined flavor producing capabilities as model systems for the study of enzymes and metabolites linked to the development of 1) desirable and 2) undesirable flavors in Cheddar cheese.

Project Summary: (Suitable for inclusion in Center documents released to the public)
Short-chain fatty acids have a role in Cheddar cheese flavor development. The mechanisms responsible for their production in cheese are not established. Microbial lipases are involved, however, lactic acid bacteria typically possess extremely weak lipolytic ability and do not produce improved flavor, even with use of strains that over express the lipase (Holland et al., 1996). Alternatively, short chain fatty acids in Cheddar cheese may arise from microbial catabolism of branched chain amino acids. The aim of this study was to determine the diversity of volatile fatty acid (VFA) production in bacteria associated with cheese processing.

All bacteria tested produced VFA in laboratory conditions with diversity in genera and species in complex laboratory media. Strains of lactococci, lactobacilli, micrococi, pediococci, leuconostoc, and brevibacteria convert amino acids to various VFA. Brevibacteria produced 10–100 times more VFA than the other bacteria tested. Studies linking carbohydrate starvation and amino acid metabolism demonstrated that some strains appeared to die by growth studies.
However, brevibacteria and lactococci maintained the ability to produce increasing amounts of VFA in carbohydrate starvation conditions in a chemically defined media (CDM), despite an apparent decrease in viable cells. Isovaleric acid alone was produced by strains in CDM at pH 7, while no fatty acids were detected in CDM at cheese-like conditions. These studies indicate amino acids are converted to VFA by cheese related bacteria. Further, they indicate that the amount of sugar present regulates these metabolic processes.

NMR studies with radiolabeled amino acids demonstrated the interconversion occurs in these bacteria. Brevibacteria produced significantly more FFA from branched chain amino acids than lactococci. Lactococci produced FFA only after the onset of carbohydrate starvation. A detailed study investigating the role of carbohydrate starvation on VFA production is under way.

Each organism produced specific FFA in cheese slurries. The relative amounts observed in the NMR studies did not match the amounts produced in slurries. The slurries did not contain other contaminating organisms, indicating that other organisms were not responsible for the increase. Alpha-keto acids are required for the conversion. However, these compounds also degrade to VFA’s at the pH and salt content of cheese. Further work is needed to define the mechanisms associated with the increases.

New knockout mutants from J. Steele (Wisconsin) and M. Yvon (France) that deleted the aminotransferase enzyme demonstrate the type of VFA produced is different when compared to the wild type. This indicates that multiple metabolic pathways exist for production of VFA in lactococci. This enzyme is one route to initiate catabolism of amino acids. This will allow a hypothesis for the catabolic pathway in cheese. Further screening for volatile fatty acids are underway.

1. Significant Progress against Objectives:
All objectives are on schedule as listed in the proposal

2. Significant Conclusions:
Dairy related bacteria convert amino acids to fatty acids that are not found in milk fat. The implications for cheese flavor indicate the starters, adjunct, and NSLAB bacteria produce VFA.

3. Anticipated Problems/Delays:
Due to personnel changes the project has been delayed in defining the observations in slurries. Recent arrival of a new MS student has this project back on track. Slurries or cheese production will begin by August.

Rapid progress is being made in selecting strains for use in cheese slurries and defining the mechanism of catabolism.
Publications:
1 in preparation

Theses:
B. Ganesan – In progress

Published Abstract:
none

Presentations:

Patent/Invention Disclosures:

**Technology Transfer Activities**
For information on licensing contact:
Bart Weimer (435) 797 3356

Visitors Hosted:
Paul Cihak – IFF
Vaughan Crow – NZDRI
Chakra Wijesundera – Food Science Australia
Western Dairy Center
Project Report
Reporting Period July 1, 1999 — December 31, 1999

Principal Investigators: Bart Weimer, Utah State University
Co-Investigators: Carl Brothersen, Utah State University
                  Paul Grossi, Utah State University

Project Title: Determination of the oxidation/reduction potential of cheese

Institution’s Project #: 99207

Project Completion Date: 6/30/00

National Research Plan (1997): Priority: 4; Goal: 4; Tactic: 1

Modifications to Project/Budget: None

Project Objectives: (Include any revisions to objectives)

Hypothesis
The redox potential has an impact on cheese flavor development, but due to lack of adequate analysis methods the role of residual oxygen has not been delineated.

Objectives
1. Evaluate commercially available probes for use in milk and milk products (Brothersen).

2. Use the methods to determine the redox potential of cheese slurries made with direct acid and lactic acid bacteria.

Project Summary: (Suitable for inclusion in Center documents released to the public)

1. Significant progress against objectives:

2. Significant Conclusions:

3. Anticipated Problems/Delays:
Publications:

Theses:

Published Abstract:

Presentations:

Patent/Invention Disclosures:

Technology Transfer Activities

Visitors Hosted:
Western Dairy Center
Project Report
Reporting Period July 1, 1999 — December 31, 1999

Principal Investigators: Mark McGuire, University of Idaho
Michelle McGuire, Washington State University
Kathy Beerman, Washington State University

Project Title: Effect of butter and margarine intake on human milk CLA and fat concentrations in lactating women.

Institution's Project #: 99206
BGK792 (UI), 2157-0018 (WSU)

Project Completion Date: 6/30/2000

National Research Plan (1997): Priority: 4; Goal: 4; Tactic: 1

Modifications to Project/Budget:

Project Objectives: (Include any revisions to objectives)

Project Summary: (Suitable for inclusion in Center documents released to the public)
1. Significant Progress against Objectives:

We have made the following progress toward meeting our objectives for this project. First, we have commitment from Ms. Nicole Andersen, a graduate student in the Department of Food Science and Human Nutrition at Washington State University, to conduct the experiment as part of her master’s program. She is being advised by Drs. Shelley McGuire, Kathy Beerman and Mark McGuire. During the fall semester, we worked on revising and expanding the protocol so that the design and methods are better outlined and described. In summary, the experiment will be 32 d in length with 6 periods: baseline (3 d), intervention I (5 d), washout I (7 d), intervention II (5 d), washout II (7 d) and intervention III (5 d). Interventions will consist of enriching the diets of the women with butter (containing CLA and no industrially-produced \textit{trans} fatty acids), regular margarine (containing no CLA and high amounts of industrially-produced \textit{trans} fatty acids) and no trans margarine (containing either CLA nor industrially-produced \textit{trans} fatty acids). We will be collecting blood and milk samples on the last day of each period, dietary information on the last 3 d of each intervention period, milk output data on the penultimate day of each intervention period, maternal and infant weight data before and after the study and maternal body fat estimations (via dual energy x-ray absorptiometry) during the baseline period (see Figure 1).

We have also worked on the development of snacks containing 50 g of either butter or one of the margarines that we will use. Further, we have set up a collaboration with Dr. Sheila Innis (University of British Columbia, Vancouver) regarding the use of a \textit{trans} fatty acid database she has developed. Currently, we are completing the forms required for both the University of Idaho and Washington State University Institutional Review Boards (IRB). We anticipate that these will be approved within a month. Recruitment will begin immediately upon getting IRB approval, and we anticipate that subject enrollment will continue throughout the summer with biochemical analyses occurring both during the summer and fall.

2. Significant Conclusions:

None to date.

3. Anticipated Problems/Delays:

Because of some initial difficulty that we had in fiscal transfers between the University of Idaho and Washington State University as well as the time required to find study personnel and revise and expand the initial protocol, we anticipate that we will not have this project completed by this summer and request that the spending of funds be extended until February 1, 2001. This will give us the time required to fully analyze the data both biochemically and statistically.
Publications:
None to date.

Theses:
None to date.

Published Abstract:
None to date.

Presentations:

Patent/Invention Disclosures:
None to date.

| Technology Transfer Activities |

Visitors Hosted:
None to date.
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Figure 1. Schematic describing sampling for Western Dairy Center (McGuire - UI).
Millions of people worldwide suffer from some type of irregular digestive tract inflammatory disease including ulcers, colitis and inflammatory bowel disease (IBD). Based on the prevailing notion that Lactobacillus, a bacterium found in many dairy products, provides good intestinal health we will investigate the possibility that Lactobacillus can modulate the inflammatory process observed in a mouse model for the human disease of spontaneous IBD. During the progression of an inflammatory response certain blood cells become activated and secrete proteins called cytokines which induce inflammatory responses. We will test whether Lactobacillus deregulates production of the cytokines or prevents activation of these inflammatory blood cells. Mice will be fed Lactobacillus in milk as the disease progresses. Many pathological and immunological parameters will be monitored during these experiments. The goal is to investigate whether Lactobacillus can inhibit the inflammatory response observed during IBD. These experiments may provide the basis for studies in humans showing that dairy product consumption or dairy products with Lactobacillus as adjuncts may inhibit the progression of IBD. The specific aims for this proposal are listed below.

1. Significant Progress against Objectives: In 2 experiments we found that probiotic treatment inhibited death of the LPS treated mice. This supports the contention that probiotics may be used to treat inflammatory based diseases
2. Significant Conclusions: Probiotic treatment of mice can suppress inflammatory reactions.

3. Anticipated Problems/Delays: There is a delay in receiving mice.

Publications: None

Theses: None

Published Abstract: None

Presentations: None

Patent/Invention Disclosures: None

Technology Transfer Activities
For information on licensing contact:

Visitors Hosted: None
Western Dairy Center
Project Report
Reporting Period January 1, 1999 - December 31, 1999

Principal Investigators: Scott Rankin, University of Maryland
Co-Investigators: Jeff Broadbent, Utah State University

Project Title: Confirm the action of key enzymes in cheese slurries and low-fat Cheddar cheese

Institution's Project #: 97087

Project Completion Date: 12/31/99 (no-cost extension to June 30, 2000)

National Research Plan (1997): Priority: 1; Goal: 1; Tactic: 2

Modifications to Project/Budget:
No-cost extension to June 30, 2000

Project Objectives: (Include any revisions to objectives)
Confirm the action of key enzymes in cheese slurries and in low-fat Cheddar cheese

Project Summary: (Suitable for inclusion in Center documents released to the public)
1. **Significant Progress Against Objectives:** Fundamental to the understanding of pathways involving aromatic amino acids is the determination of which reactions are enzymatically catalyzed and which result from spontaneous degradation. To this end we have secured all readily available degradation products of the amino acids tyrosine and phenylalanine. Additionally we have developed a method of suspending amino acid products of varying solubility in model cheese systems. In short, each compound is suspended in a sterile aqueous model cheese system and analyzed for spontaneous degradation over time. Compounds currently under scrutiny include: tyrosine, p-hydroxy phenylpyruvate, p-hydroxyphenylacetate, p-cresol, phenol, phenylalaninc, phenacetate, phenylacetaldehyde, benzylacetate, ethylbenzene, 2-phenylethanol, β-phenylpyruvic acid.

2. **Significant Conclusions:** Progress has been slowed due to some methodology setbacks. We are in the process of resolving those setbacks and have requested a 6-month no-cost extension.

3. **Anticipated Problems/Delays:** As noted above, the methodology originally proposed has shown limitations to these objectives. Because we have expanded the compound stability study beyond p-cresol, many of the compounds are not volatile, hence unresolvable using gas chromatography. We have secured an HPLC system with an appropriate column and UV detector. The HPLC operating parameters are currently being optimized for the desired sensitivity and precision with the cheese model system analytes.

**Publications:** None

**Theses:** None

**Published Abstract:** None

**Presentations:** None

**Patent/Invention Disclosures:** None

| Technology Transfer Activities | None |

**Visitors Hosted:** None
Western Dairy Center
Project Report
Reporting Period January 1, 1997 — December 31, 1999

Principal Investigators: Bruce L. Geller, Oregon State University
Co-Investigators:

Project Title: Lactococcal Nasal Vaccine
Institution’s Project #: 98096
Project Completion Date: 12/31/99

National Research Plan (1997): Priority: Goal: Tactic:

Modifications to Project/Budget:
None

Project Objectives: (Include any revisions to objectives)
1) To construct strains of Lactococcus lactis that express on their cell surface the M6 protein from Streptococcus pyogenes. 2) To vaccine cows by nasal administration of the live, recombinant strains of L. lactis made in objective #1. 3) To measure by ELISA assay the mucosal immune response in the milk of the vaccinated cows.

Project Summary: (Suitable for inclusion in Center documents released to the public)

1. Significant Progress against Objectives:

A strain of Lactococcus lactis was genetically engineered to express the receptor binding site of M6 protein (M6c) from Streptococcus pyogenes. The level of expression of M6c was increased approximately 25-fold by substituting the native lactococcal promoter with a stronger promoter (pLA16) from Lactobacillus. The proportion of M6c attached to the surface or secreted into the culture supernatant of L. lactis varied with the amount expressed. At low levels of expression, about 75% of M6c was attached to the surface of L. lactis and the remainder was found in the culture supernatant. At the highest level of expression with the pLA16 promoter, about 75% was secreted into the medium and 25% was firmly attached to the cell surface.

I arranged for the use of 16 cows at the OSU dairy barn for vaccination.
Unfortunately, my contact with the Oregon dairy farmers was unable to secure and deliver the hay required to feed the cows. Because of this, it was decided to test the vaccine in mice before using cows.

Mice were vaccinated nasally with either a strain of *L. lactis* that expressed the M6c antigen or an isogenic control strain that did not express the foreign antigen. In a second experiment, a nasal adjuvant, CpG, was co-administered with the vaccine strains. The vaccinated mice were monitored by ELISA for anti-M6c antibodies in their blood serum and saliva before and after vaccination. The results indicated no significant difference in serum or saliva anti-M6c antibodies between the M6c- and control-vaccinated groups.

The vaccinated mice were challenged with an oral dose of *S. pyogenes*. Throat swabs of each mouse were taken before and after the challenge, and are a measure of infection. We found that the number of infected animals was not significantly reduced by vaccination with the M6c-producing strain (Table 1).

Table 1. Numbers of mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Challenged</th>
<th>Infected</th>
<th>Infected &amp; died</th>
<th>Infected &amp; not die</th>
<th>Infected &amp; cured</th>
<th>Not infected, died</th>
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<tr>
<td>Control</td>
<td>18</td>
<td>4</td>
<td>4</td>
<td>0</td>
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<td>M6c</td>
<td>19</td>
<td>13</td>
<td>7</td>
<td>6</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Control + CpG</td>
<td>20</td>
<td>12</td>
<td>7</td>
<td>5</td>
<td>2</td>
<td>1</td>
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<tr>
<td>M6c + CpG</td>
<td>20</td>
<td>12</td>
<td>5</td>
<td>7</td>
<td>4</td>
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<tr>
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<td>16</td>
<td>7</td>
<td>9</td>
<td>5</td>
<td>1</td>
</tr>
</tbody>
</table>

2. Significant Conclusions:

1. M6c protein was expressed from *L. lactis*. Most of the secreted into the medium, although about 25% was firmly attached to the cell surface.
2. Nasal vaccination of mice with the strain of *L. lactis* producing M6c did not increase specific serum or salivary antibodies to M6c.
3. Mice vaccinated with the strain of *L. lactis* producing M6c were not protected against infection by *S. pyogenes* or death.
4. Mice vaccinated with the strain of *L. lactis* producing M6c were not more likely to be cured after becoming infected with *S. pyogenes* than mice vaccinated with the negative control strain.

3. Anticipated Problems/Delays:
Publications:


Theses:

Published Abstract:

Presentations:

Patent/Invention Disclosures:

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<th>Technology Transfer Activities</th>
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Visitors Hosted:
Western Dairy Center
Project Report
Reporting Period January 1, 1997 – December 31, 1999

Principal Investigators: Bruce L. Geller, Associate Professor of Microbiology, Oregon State University

Co-Investigators:

Project Title: The Use of Bacteriophage-Receptor Genes of Lactococcus lactis to Develop Bacteriophage Resistance in Cheddar Cheese Starter Strains

Institution’s Project #: 97081

Project Completion Date: 12/31/00

Understand phage-resistance systems of starter cultures and phage counter defense systems to develop longer lasting phage resistance strategies.

Modifications to Project/Budget:
One year no-cost extension to 12/31/00

Project Objectives: (Include any revisions to objectives)
1. Identify and isolate host genes other than pip that are required for infection by phage of the c2 species. 2. Identify a gene encoding a receptor for a phage of the p335 species. 3. Identify a gene encoding a receptor for a phage of the 936 species. 4. Construct a phage-resistant strain of the L. lactis with defective copies of the receptor genes for phages of the c2, 936 and p335 species. 5. Evaluate the effects of receptor gene mutations on phage resistance, growth rate, acidification and coagulation of milk, and other cheese production traits.
**Project Summary:** (Suitable for inclusion in Center documents released to the public)

The proposed research examines early steps of bacteriophage infection of *L. lactis*, which include attachment of the phage to the surface of cells and entry of phage DNA into cells. Both of these steps are required for infection by phage. Our strategy of strain improvement is to prevent the phage from attaching or entering the host in the first place. To do this requires knowledge of the host components required for attachment and phage entry. The outcomes of this proposal will enable the construction of new strains with defined mechanisms of phage-resistance. Host genes required for phage infection of *L. lactis* will be identified and isolated. We have previously isolated one such gene named pip (an acronym for phage infection protein). The protein encoded by *pip* (Pip) is a receptor for phage attachment and phage DNA entry into the host. We have constructed phage-resistant strains of *L. lactis* by replacing the pip gene with a defective version. There is evidence that host components in addition to pip are required for phage attachment and DNA entry. Isolating genes in addition to pip that are required for phage infection will enable the construction of new strains with alterations in two or more different host components. The strategy of combining multiple phage-resistance mechanisms will greatly decrease the chance that the strain will fail after introduction into commercial use. Genes will be isolated that also extend the range of resistance to phages that do not require pip. We propose to isolate genes that encode host receptors for two different types of small isometric-head phage (p335 and 936). Together with the phages that required pip, the p335 and 936 species of phage cause nearly all the starter failures in U.S. cheese factories. Phages of the p335 species are particularly troublesome, as they have only recently emerged as a major problem, and less is known about their mechanism of infection. The isolated receptor genes will be inactivated and used to construct a new commercial strain with a combined phage-resistance defined by each of the inactivated genes (including pip). The phage-resistant strain will be evaluated for physiological characteristics important for making cheese.

1. **Significant Progress against Objectives:**

   We have cloned by complementation, lactococcal genes required for infection of phage sk1, which is a phage of the species 936. Phage-resistant mutants of *L. lactis* C2 were isolated after challenge of *L. lactis* C2 with page sk1. One resistant strain, RMSK1/1, had cell wall compositions indistinguishable from that of *L. lactis* C2 and adsorbed phage sk1 to the same extent as strain C2. RMSK1/1 has characteristics consistent with a strain mutated in its cell membrane receptor.

   To identify the locus (or loci) of *L. lactis* responsible for this resistance to phage sk1, RMSK1/1 was transformed with a genomic library of *L. lactis* LM2301 DNA (LM2301 is a plasmid-less derivative of C2). Transformants were screened for phage sensitivity. Two transformants with overlapping DNA that complemented RMSK1/1 to phage sensitivity were analyzed.

   We have completely sequenced the cloned DNA, and identified all open
reading frames by homologies to known genes in the databases. The cloned DNA includes an open reading frame with similarity to lysozymes (β-1,4-N-acetylmuramidase) of phage of *Lactobacillus* and *Streptococcus*, DNA homologous to non-coding sequences of temperate phage of *Lactococcus lactis*, a gene with similarity to tRNA genes, a prophage attachment site, and open reading frames with similarities to *sun*, phosphoprotein phosphatases, and protein kinases.

The lysozyme gene has been designated *lysL*. Lysozymes degrade bacterial cell walls, and bacteriophage often encode their own lysozyme. Indeed, LysL is similar to lysins of virulent phage LL-H of *Lactobacillus delbrueckii* subsp. *lactis*, Cp-1 and Cp-9 of *Streptococcus pneumoniae*, and of temperate phage mv4 of *Lactobacillus delbrueckii* subsp. *lactis*, and phage adh of *Lactobacillus gasseri*.

Starting 307 bp distal to *lysL* was DNA similar to that of temperate lactococcal bacteriophage of the p335 species, including BK5-T, rlt, lc3, and Tuc2009. These regions of the phage genomes contain sequences involved in transcription termination and homologous recombination and are from 173 to 197 bp distal to the respective lysin genes. An alignment of these similar nucleotide sequences revealed that this region of *L. lactis* DNA similar to temperate phage of the p335 species contains a 266 base-pair stretch of DNA that interrupts the region of similarity. The 266 base-pair intervening sequence contains a set of inverted repeats at the borders, and another set in the exact middle of the sequence. The symmetry of the inverted repeats in this 266 bp sequence suggests that a transposition event occurred in this region.

Several lines of evidence indicate that this region does not contain an intact integrated prophage, despite the fact that lysogeny is the rule rather than the exception for lactococcal strains. Indeed, strain C2, the parent of strains RMSK1/1 and LM 2301, harbors a prophage that produces a small, isometric virion upon induction with UV light. Although DNA similar to that of temperate lactococcal bacteriophages of the p335 species was detected distal to *lysL*, the lysin enzymes of these temperate phages are less similar to LysL than the proteins discussed above. In addition, the regions bordering *lysL* were not similar to the att regions of temperate phages, and the regulatory sequences present in lytic and temperate phages of *Lactococcus lactis* were not present in the 5832 bp sequenced. Also, the region of similarity to temperate phage of the p335 species appeared to be interrupted by a transposition event. The homologies of *lysL* with lysin genes from other phages, and the similarities with regions of temperate phage of the p335 species suggests that this may be the site on an ancient propane integration.

Starting from the end of *lysL* (nucleotide 1169) to nucleotide 2209, there is a 42% identity with the phage sk1 genome from nucleotides 22109 through 23198. This region of the phage genome encodes 4 orfs of unknown function, the early promoter E5, and the 3' end of a putative phage DNA polymerase subunit. Nucleotides 2051 through 2154 of the cloned DNA are 58% identical to phage sk1 DNA from nucleotides 17501 through 17604. This later region of the phage DNA encodes the early promoter E6 and a partial orf of unknown function.

Beginning at nucleotide 2209 through 2258 is DNA with similarities to tRNA genes. An analysis of secondary structures revealed a lack of a consensus anticodon stem structure, suggesting that this region does not code for a functional tRNA.
Distal to the tRNA gene starting at nucleotide 2313 is 13 bp of DNA that is 85% identical to the attP of the temperate p335 species bacteriophage TP901. Distal to attP are orfs with similarities to sun, phosphoprotein phosphatases, and protein kinases. These genes were not studied further, as they were not involved with complementation of phage sk1-resistance.

We have found that the non-coding DNA with similarity to temperate phages and phage sk1 was responsible for complementation. Complementing DNA was subcloned, using phage sensitivity to assay for complementation. The region responsible for the complementation was narrowed to lysL and about 1300 bp downstream of this locus. The region further downstream (including the tRNA-like gene, attP, and all orfs except lysL) did not complement. We constructed a modified version of lysL that contains nonsense codons in all three frames and a unique restriction site as a distinguishing marker. This nonsense allele complemented the phage resistant mutant. In addition, we exchanged the wild type lysL in the phage-sensitive strain LM2301 with the nonsense allele and found that the strain was still phage sensitive. We conclude that lysL is not involved in the phage-resistance mechanism.

The above results suggested that the region downstream of lysL was responsible for complementation. We deleted this region and found that complementation in trans was eliminated. We also deleted this region from the chromosome of strain LM2301 and found that it was not phage resistant. We conclude that the non-coding region with similarity to temperate phages and phage sk1 was responsible for complementation. We also conclude that deletion of this complementing region does not cause phage sk1 resistance. This suggests that phage resistance in RMSK1/1 is caused by an unidentified mechanism of resistance.

To learn more about this unusual mechanism of resistance, we further characterized strain RMSK1/1. The frequency of cell death upon addition of phage sk1 to liquid cultures of RMSK1/1 was investigated. We found no difference in the number of viable cells or growth rate in the presence or absence of phage sk1. This suggests that phage sk1 DNA does not enter RMSK1/1. This also suggests that the mechanism of resistance is neither abortive infection nor restriction/modification.

The ability of RMSK1/1 to replicate phage sk1 DNA and assemble and release mature phage particles was analyzed. Phage sk1 DNA was introduced to strains RMSK1/1, LM2301, and C2 by electroporation. The results show that RMSK1/1 produced phage from phage sk1 DNA as well as strains LM2301 and C2. We conclude that phage sk1-resistance in RMSK1/1 is not an abortive infection mechanism.

The efficiency of plaquing (EOP) was analyzed. Previously, we had found that the EOP on strain RMSK1/1 was undetectable. This was confirmed by plating 10^7 PFU and observing no plaques on RMSK1/1. However, we observed that the transformant RMSK1/1 (pSA3) formed plaques at an EOP of 0.1 when the calcium concentration in the growth agar was increased to 10 mM from our usual concentration of about 1 mM. The diameter of plaques formed on RMSK1/1 (pSA3) was estimated at about 0.1 mm (pinpoint).

It was hypothesized that the slower growth rate of RMSK1/1 (pSA3) compared to RMSK1/1 was one factor that enabled plaques to form. This was
tested by titrating phage sk1 on RMSK1/1 at growth temperatures of either 30°C or 20°C, including 10 mM calcium in the agar. The results were that the EOP was undetectable (no plaques) at 30°C and was 0.1 at 20°C.

Plaques formed on RMSK1/1(pSA3) did not form plaques on RMSK1/1, nor did the EOP increase after passage through RMSK1/1 (pSA3) or RMSK1/1 at 20°C. We conclude that the mechanism of resistance of RMSK1/1 is not restriction/modification.

Adsorption of φsk1 to isolated plasma membranes was measured in vitro. Purified plasma membranes from either RMSK1/1 or LM 0230 were mixed and incubated with φsk1. Membranes from either strain inactivated the phage, indicating that there is no defect in adsorption to the plasma membrane in RMSK1/1. Phage adsorption was not inhibited by rhamnose or N-acetylglucosamine (two inhibitors of φsk1 adsorption to the cell wall receptor), indicating that phage inactivation was not caused adsorption to the cell wall carbohydrate receptor. Moreover, non-specific adsorption cannot account for this result, because purified plasma membranes from Escherichia coli did not inactivate φsk1.

Collectively, the above results indicate that the mechanism of phage resistance of RMSK1/1 is an inhibition of phage DNA injection into the host cell. This mechanism may be similar to one described recently by Gerald Fitzgerald's group for a strain resistant to c2 species phage.

We have also constructed insertion mutants of Lactococcus lactis that are resistant to a p335 species phage. We have screened 6 of these mutants and found that they are defective in absorbing phage. We plan to clone at least one of the genes responsible for the phage-resistant phenotype. However, progress on this goal has been on hold since 1998 due to lack of personnel to complete this part of the project. It is anticipated that a graduate student will continue this work in 2000 and 2001.

2. Significant Conclusions:

A phage sk1-resistant mutant of L. lactis strain C2 was restored to phage-sensitivity by complementation with cloned host DNA. Characterization of the complementing DNA revealed a gene that encodes a protein related to lysins of phage of Lactobacillus spp and Streptococcus pneumoniae. Distal to this gene is DNA homologous to phage sk1 and non-coding sequences of temperate phage of Lactococcus lactis. These similarities suggest that this phage-sensitivity region may have originated as part of a prophage.

The DNA with similarities to non-coding regions of temperate phage and phage sk1 was responsible for complementation.

3. Anticipated Problems/Delays:

The PI, Bruce Geller is on sabbatical leave from August 1999 to August 2000, and was granted by Bob Champion (DMI) a one year, no-cost extension to continue the project. It is anticipated that additional time will be required after 12/31/00 to complete the project because of the delay caused by the sabbatical leave.
Publications:


Theses:
None

Published Abstract:
None

Presentations:
None

Patent/Invention Disclosures:
We have filed an Invention disclosure with the Technology Transfer Office at Oregon State University. We have signed confidentiality agreements with 3 major culture suppliers, with the objective of negotiating an agreement with one or more of them for licensing this invention. The results suggest that RMSK1/1 contains a unique mechanism of resistance for 936 phages, which may be of interest for commercial development.

Technology Transfer Activities
For information on licensing contact:

Visitors Hosted:
None