6-30-2000

2000 Semi-Annual Report

Various Authors

Western Dairy Center

Project Report

Reporting Period June 1, 1997 — June 30, 2000

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 byUnderstanding 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 poysaccharide production by starter cultures in cheese quality and functionaity.

Modifications to Project/Budget:
None

Project Objectives: (Include any revisions to objectives)

1. Characterize the structure, molecular weight, and polymer properties of the exopolysaccharide produced by *S. thermophilus* MR-1C.
2. Isolate and characterize the *S. thermophilus* MR-1C gene cluster for exopolysaccharide production.
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. We have also completed nucleotide sequence analysis for a region encompassing 36 kb of the MR-1C chromosome that contains the *cps* gene cluster. Capsule production in this bacterium appears to involve at least 19 different genes. Like each of the 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 product that is 99% identical to the *S. thermophilus* Sfi6 EpsE protein, a phosphogalactosyl-transferase 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 require the identification of MR-1C *cps* genes that are essential for capsule production. To address this need, we have initiated work to identify regions of the MR-1C *cps* cluster that are not present in the industrial, fast acid-producing, EPS-negative, Mozzarella cheese starter bacterium *S. thermophilus* TAO61. Preliminary analysis of MR-1C and TA061 indicates that both contain the 4-kb *epsA-D* gene regions found in other *S. thermophilus* strains. Polymerase Chain Reaction (PCR) analyses also indicate the presence of genes similar to the MR-1C *cpsL* and *cpsU* genes in TA061, as well as sequences that lie immediately downstream of the MR-1C

cps gene cluster. Thus, TA061 does appear to possess at least some homologs to MR-1C *cps* genes. Sequence analysis of the available TA061 PCR fragments revealed that overall they are 94 to 99% identical to the corresponding MR-1C regions. The greatest difference identified so far is in the *cpsC* gene where TA061 is only 86.2% identical to MR-1C. This difference may be significant since the *cpsC* gene is predicted to be important in determination of the chain length of the polysaccharide.

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 ($P < 0.01$) than whey from cheeses made with MR-1C, TAO61, 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:

Broadbent, J.R., D.J. McMahon, C.J. Oberg, and D. L. Welker. Use of exopolysaccharide-producing cultures to improve the functionality of low fat cheese. Int. Dairy J. (submitted)

Petersen, B. L., R.I. Dave, D.J. McMahon, C.J. Oberg, and J.R. Broadbent. 2000. Influence of capsular and ropy exopolysaccharide-producing *Streptococcus thermophilus* on Mozzarella cheese and cheese whey. J. Dairy Sci. 83:(in press).

Oberg, C.J., J.R. Broadbent, and D.J. McMahon. 1998. Developments in thermophilic starter cultures for cheese. Aust. J. Dairy Technol. 53:102-104.

Low, D., Ahlgren, J.A., D. Horne, D.J. McMahon, C.J. Oberg, and J.R. Broadbent. 1998. Role of *Streptococcus thermophilus* MR-1C capsular exopolysaccharide in cheese moisture retention. Appl. Environ. Microbiol. 64:2147-2151

Theses:

Low, D. 1998. Influence of Streptococcus thermophilus MR-1C capsular exopolysaccharide on moisture level of low-fat Mozzarella cheese. MS thesis, Utah State University.

Published Abstract:

Peterson, B.L., R.I. Dave, D.J. McMahon, C.J. Oberg, and J.R. Broadbent. Influence of capsular and ropy exopolysaccharide-producing *Streptococcus thermophilus* on Mozzarella cheese and cheese whey. J. Dairy Sci. 82(Suppl. 1):2.

Low, D., D.J. McMahon, C.J. Oberg, D. Horne, and J.R. Broadbent. 1997. Influence of *Streptococcus thermophilus* 10JC exopolysaccharide on the moisture content of low-fat Mozzarella cheese. J. Dairy Sci. 80(Suppl. 1):107.

Presentations:

Broadbent, J.R. 2000. Use of exopolysaccharide-producing cultures to improve the functionality of low fat cheese. Oral presentation at International Dairy Federation Symposium on Ripening and Quality of Cheeses. March 12-17, Banff, Canada.

Peterson, B.L., R.I. Dave, D.J. McMahon, C.J. Oberg, and J.R. Broadbent. 1999. Influence of capsular and ropy exopolysaccharide-producing *Streptococcus thermophilus* on Mozzarella cheese and cheese whey. Oral presentation at the Ann. Mtg. Amer. Dairy Sci. Assoc. June 20-23, Memphis, TN.

Broadbent, J.R. 1999. New Cultures. Invited oral presentation for the International Dairy Foods Association Cultured Dairy Products Conference. May 11-12, Milwaukee, WI.

Broadbent, J.R., D.J. McMahon, and C.J. Oberg. 1998. Practical Considerations in the Use of Exopolysaccharide-producing Cultures. Invited oral presentation for the Marschall/Rhodia, Inc. Italian and Specialty Cheese Conference, Sept. 16-17, Madison, WI.

Broadbent, J.R. 1998. Role of the *Streptococcus thermophilus* exopolysaccharide in cheese moisture retention. Invited oral presentation for Texel International. April 30, Dange, France.

Broadbent, J.R., D. Low, D., D.J. McMahon, C.J. Oberg, and J.A. Ahgren. 1998. Properties of a novel *Streptococcus thermophilus* capsular heteropolysaccharide. Poster presentation at the American Society for Microbiology's Conference on Streptococcal Genetics. April 26-29, Vichy, France.

Broadbent, J.R. 1997. EPS production in *Streptococcus thermophilus*: physiology, biochemistry, and genetics. Invited oral presentation for the Marschall/Rhône-Poulenc Italian and Specialty Cheese Conference, Sept. 17-19, Madison, WI.

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

Visitors Hosted:

none

Western Dairy Center Report Addendum

Confidential - For Center Director's Use Only
(Release of this information requires prior approval by principal investigator)

Provide summary of significant advances in project that you wish to remain confidential at this time. For example, new methodology that you do not with to release until the current work is completed; information on invention disclosures or patents filed; activities conducted to transfer the technology to industry, or licensing agreements that are underway.

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:
The MR-1C culture has been provided to 3 different culture companies for testing. None has committed to a commercial licensing agreement.

Discoveries:
Commercial-scale (22,000 kg vats) testing of the water-binding properties of MR-1C was performed by the Gist-Brocades (Millville, UT) culture company. Those experiments confirmed that part-skim Mozzarella cheese made with MR-1C and an EPS- rod contained 1.5% more moisture than cheese made with a conventional EPS- Mozzarella starter pair. These data demonstrate that MR-1C can be used to increase cheese yield under the constraints posed by industrial-scale manufacture.

Research has also been performed to address a common concern over the use of EPS^+ cultures in cheese. Several companies have expressed interest in the ability of our encapsulated *Streptococcus thermophilus* strain, MR-1C, to significantly increase moisture retention in low-fat Mozzarella cheese. Their willingness to use of EPS^+ cultures in cheese, however, has been tempered by the fear that this culture will increase whey viscosity, thus retarding the efficiency of whey concentration and drying, and altering the functional properties of whey products. In order to address these concerns, we examined the effect of capsular and ropy *S. thermophilus* starter bacteria on Mozzarella cheese and whey. Cheeses were manufactured 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 ($P < 0.01$) than whey from cheeses made with MR-1C, TAO61, 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 clearly show that encapsulated (but not ropy) EPS^+ *S. thermophilus* can be used to increase cheese moisture levels without affecting whey viscosity.

Another significant discovery in this work relates to galactose catabolism by *S. thermophilus*. Residual galactose in Mozzarella cheese can promote Maillard browning during high-temperature cooking of pizza, and this reaction can be problematic because excess browning makes the product less appealing to consumers. For this reason, the capability to metabolize galactose is a desirable characteristic in Mozzarella starter cultures, but most strains of *S. thermophilus* and *Lactobacillus bulgaricus* cannot metabolize the galactose moiety of lactose. Instead, these bacteria excrete galactose into the medium via an antiport system that drives lactose uptake. This is one reason that many processors now use *Lactobacillus helveticus* strains, which are able to ferment residual galactose, instead of *L. bulgaricus* in Italian starter blends. Although *L. helveticus* do help to reduce browning, even better control over nonenzymatic might be possible if a galactose-fermenting coccus was included with the rod. One of the unexpected results from our EPS study was the discovery that the EPS-negative mutant, DM10, had acquired the ability to ferment galactose. The reasons for this ability are unclear at the moment, but it is known that even though most strains of *S. thermophilus* are unable to ferment galactose, they do possess genes encoding the Leloir enzymes for galactose catabolism. This finding has fueled speculation that in *S. thermophilus*, Leloir enzymes may function primarily in the production of uridine diphospho sugar precursors for EPS biosynthesis, instead of galactose breakdown. Nevertheless, one study has reported that Gal^+ *S. thermophilus* can be obtained by mutations in the *gal* promoter region which increased transcriptional activity, and suggested that the Gal- phenotype of this bacterium was due to very low level expression of Leloir enzymes. Since the gene we inactivated in DM10, *cpsE* , encodes the galactosyltransferase that catalyzes the first step in the assembly of the EPS basic repeating unit, which is transfer of Gal-1-phosphate from UDP-Gal to the undecaprenyl-phosphate lipid carrier, inactivation of *cpsE* may be expected to result in an accumulation of UDP-galactose within the cell. Overproduction of phosphorylated sugars can be toxic to cells, so survival and growth of a *cpsE*-negative mutant like DM10 may require a Gal^+ phenotype. Experiments are now underway to determine whether the acquisition of this phenotype was a primary (e.g. UDP-galactose was an inducer of the *gal* operon) or secondary

(e.g. coincidental mutation in the *gal* promoter region) effect of *cpsE* inactivation in *S. thermophilus* DM10. We anticipate that this research may lead to new strategies to reduce the concentration of free galactose in pizza cheese and control nonenzymatic browning during cheese baking.

Western Dairy Center

Semi-Annual Project Report

Reporting Period January 1, 2000 – June 30, 2000

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

National Research Plan (1997): Priority: Goal: 4.2 Tactic: 1,3,6
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 to 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:

Our strategy of cloning by complementation has not yielded a gene for a receptor of phage sk1 or a p335 species phage. Because of this, we thought it reasonable to identify biochemically the receptors. Using classical methods of competitive inhibition of binding to plasma membranes, we identified the membrane receptor for phage sk1. The receptor is a membrane component that differs fundamentally from the cell wall receptor.

We have also shown two other characteristics of the receptor. First, the receptor requires an interaction with the peptidoglycan for adsorption of phage. This was shown by treating plasma membrane with enzymes that hydrolyze the peptidoglycan. Following treatment with the enzymes, binding of phage sk1 to the membranes was significantly reduced. The second characteristic is that other phages of the 936 species also use the same receptor. This was shown by competitive binding between phage sk1 and other phages of the 936 species.

We think it likely that the receptor for phage sk1 is required for viability. This assumption is based on a comparison to homologous components from other bacteria. With this in mind, we designed a strategy for selection of conditional mutants. Our screening steps include a test for adsorption of phage sk1 to the plasma membrane, as well as tests for abortive infection, restriction/modification and loss of adsorption to the cell wall. We are currently in the process of screening dozens of phage sk1-resistant mutants. After the phage-resistant isolates have been characterized biochemically, we will use one for the recipient of our genomic library.

The identity of the phage sk1 receptor cannot be revealed at this time without compromising commercial interest in it by DSM Food Specialties. However, the identity has been revealed to Professor Noel Dunn and his colleagues at the Department of Biotechnology, University of New South Wales, Sydney, with whom DSM has established a mutual confidentiality agreement.

A second tactic that we are currently using to identify phage receptor genes is to characterize insertion sequence mutants. We have shown cause and effect in 13 phage-resistant mutants between the phage sk1-resistant phenotype and the presence of the insertion sequence. We are now biochemically characterizing the mutants for abortive infection, restriction/modification, and loss of adsorption mechanisms of resistance. Later this month we will clone at least part of one of the mutated genes by the method of Maguin.

A third tactic that we are using has been to construct a plasmid-free strain that is infected by three p335-species phages. We cured Lactococcus lactis strain UL8 of all but its smallest endogenous plasmid. The small plasmid that remains is about 2 kb and should not interfere with our isolation of host genes required for p335 species phage infection. The derived strain was mutated by insertion mutagenesis and selected for resistance to a p335 species phage. We are currently characterizing the mutants for the mechanisms of resistance.

2. Significant Conclusions:

- Small isometric lactococcal phages of the 936-species, including phage sk1, require a plasma membrane receptor for infection.
- The receptor has been identified by biochemical methods, and its identity has commercial value.
- The receptor is likely to be required for viability.

3. Anticipated Problems/Delays:

The PI, Bruce Geller is on sabbatical leave from August 1999 to August 2000, and was granted 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.

4. Publications:

Kraus, J. and B. L. Geller. 2000. Cloning of genomic DNA of *Lactococcus lactis* that restores phage sensitivity to a bacteriophage sk1-resistant mutant. Submitted.

5. Theses:

Hang Ngo. Identification and Characterization of the Plasma Membrane Receptor for Phage sk1 of Lactococcus lactis. University of New South Wales, 2000.

Putri Realita. Construction and Selection of a Strain for Cloning Host Genes Required for p335 Species Phage of Lactococcus lactis. University of New South Wales, 2000.

6. Published Abstract:

None

7. Presentations:

Hang Ngo. Identification and Characterization of the Plasma Membrane

Receptor for Phage sk1 of Lactococcus lactis. University of New South Wales, 2000.

Putri Realita. Construction and Selection of a Strain for Cloning Host Genes Required for p335 Species Phage of Lactococcus lactis. University of New South Wales, 2000.

8. Patent/Invention Disclosures:
The PI has signed a confidentiality agreement with DSM Food Specialties. The value of our results to DSM depends on keeping proprietary the identity of the phage sk1 receptor. DSM is interested in evaluating the use of the receptor genes in their production strains.

Technology Transfer Activities
For information on licensing contact:

Visitors Hosted:
None

Western Dairy Center

Project Report

Reporting Period July 1, 1997 — June 30, 2000

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

National Research Plan (1997): Priority: 5 Goal: 1 Tactic: 4

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

paterns 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 secuenced 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:

Numereous 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:

Pederson, J. A., G. J. Mileski, **B. C. Weimer**, J. L. Steele. 1999. Genetic Chancterization of a Cell Envelope-Associated Proteinase from *Lactobacillus helveicus* CNRZ32. J. Bact. 181:4592–4597.

Koka, R., and **B. C. Weimer**. Isolation and characterization of a protease from *Pseudomonas fluorescens* RO98. J. Applied Microbiol. (accepted).

Koka, R., M. Blake, and **B. Weimer**. Diversity of lipases of *Pseudomonas* isolated from milk: possible existence of multiple enzymes. J. Dairy Research (accepted).

Koka, R., and **B. C. Weimer**. Rapid detection of extracellular protease from *Pseudomonas fluorescens*. Int. Dairy J. (accepted).

Koka, R., and **B. C. Weimer**. Investigation of the ability of a purified protease from *Pseudomonas fluorescens* RO98 to de–bitter cheese. Int. Dairy J. (accepted).

Theses:

Paul Joseph – Ph.D. candidate

Published Abstract:

Pederson, J. A., G. J. Mileski, **B. C. Weimer**, J. L. Steele. 1999. Genetic Characterization of a Cell Envelope-Associated Proteinase from *Lactobacillus helveticus* CNRZ32. 6th Lactic acid bacteria meetings (Veldhoven, The Netherlands). Sept. '99.

Presentations:

Weimer, B. C., P. Joseph, J. Petersen, and J. Steele 2000. The proteinases of lactobacilli. International Dairy Federation Biennial Cheese Flavor Conference, Banff, Alberta.

Koka, R. and **B. C. Weimer.** 1999. *Pseudomonas* protease: influence of growth conditions on production, isolation, and characterization. Food Micro '99, Veldhoven, The Netherlands.

Koka, R. and **B. C. Weimer.** 1999. Rapid detection of extracellular protease from *Pseudomonas fluorescens*. Food Micro '99, Veldhoven, The Netherlands.

Patent/Invention Disclosures:

none

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

Visitors Hosted:

Western Dairy Center

Project Report

Reporting Period January 1, 1997 — June 30, 2000

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

National Fluid Milk Research Plan (1997): Priority: 1 **Goal:** 2 **Tactic:** 1
Research thermal and mechanical processing methods to improve sensory and color aspects of whitness, 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

Macrosphere 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 20 ml skim milk with a 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 25°C. 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 MARIE K. WALSH. INFLUENCE OF LIMITED PROTEOLYSIS WITH IMMOBILIZED OR SOLUBLE ENZYMES ON THE WHITENESS OF SKIM MILK in press for Journal of Food Biochemistry

Walsh, M.K. and X. Li. 2000 Thermal stability of acid proteases in skim milk. In press for J. Dairy Research.

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:

Technology Transfer Activities
For information on licensing contact:

Visitors Hosted:

None

Western Dairy Center

Project Report

Reporting Period July 1, 1997 — June 30, 2000

Principal Investigators: **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: 12-31-00

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. pseudoplantarum 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 was 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, 2wk, 2 mo, 4 mo, and 6 mo cheese isolates. Isolates which displayed a unique RAPD fingerprint were 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 casei* 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. RAPD and 16S rRNA studies showed that:

1. The NSLAB biota in both cheeses changed over time, but NSLAB populations in Colby cheese retained a greater degree of heterogeneity than those of Cheddar.

2. The *Lb. casei* Lila adjunct did not dominate the NSLAB populations beyond 3 mo, but it's use did limit the heterogeneity of the NSLAB population in Colby and in young Cheddar.

3. All cheese finished with a NSLAB population dominated by more than 1 strain of *Lb. casei*. Interestingly, the dominant strains of *Lb. casei* in each cheese appeared to be most affected by adjunct treatment and not cheese variety.

3. Anticipated Problems/Delays:

none

Publications:

none

Theses:

none

Published Abstract:

none

Presentations:

Broadbent*, J.R. Role of lactic acid bacteria in cheese flavor development. Invited oral presentation for special ADSA pre-meeting workshop on the basics of flavor development in cheese. Sponsored by the American Dairy Science Association and Rhodia, Inc. July 24, Baltimore, MD.

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 — June 30, 2000

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: 12-31-00

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

1. Define the contribution of starter CEP specificity on peptide pools and bitterness in Cheddar cheese.
2. Develop a cheese-based test for bitterness in Cheddar cheese and establish factors that influence sensory perception of bitterness in Cheddar cheese.
3. Determine bitter taste thresholds for β-CN (f193-209) and α_{S1}-CN (f1-9).
4. Define the contribution of *Lactobacillus helveticus* CNRZ32 peptidases to degradation of β-CN (f193-209) and α_{S1}-CN (f1-9).
5. Construct *L. lactis* SK11 derivatives with enhanced activity of peptidases demonstrated to be important in hydrolysis of β-CN (f193-209) & α_{S1}-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-flavored Cheddar has lent greater significance to the impact of bitterness on dairy 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 by degraded by intracellular peptidases from starters and adjunct 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 the lactococcal 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 α_{S}-CN (f1-23) at pH 5.2 in 4% 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 USU Institutional Review Board (for experiments with human subjects). 50% 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 specficity of each starter. Trained sensory analysis of the experimental cheees after 2, 4, and 6 mo of ripening has established a clear role for CEP specficity 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 findings that starter culture proteinase specificity is a key determinate 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 bitteness in cheese have not yet been identified. In the past, researchers seeking 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 system, 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 compare 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 enzymes 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 α_{S1}-CN (f1-7), suggesting either that an endopeptidase distinct from PepO and PepE or a carboxypeptidase was responsible for the formation of this peptide. Therefore, Objective 5 was delayed to identify endopeptidase essential for the hydrolysis of _-CN (f193-209). To date, we have cloned and sequenced an endopeptidase O2 (*pepO2*), which has shown the capability to hydrolyze a carboxyl-blocked form of β-CN(f193-209). Next, the expression of *pepO2* in *Lc. lactis* will be examined using a transcriptional fusion vector and the enzyme will be characterized.

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 α_{S1}-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 α_{S1}-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 (f 193-209) 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:

Christensen, J.E., E.G. Dudley, and J.R. Pederson, J.L. Steele. 1999. Peptidases and amino acid catabolism in lactic acid bacteria. Antonie van Leeuwenhoek 76:217-246.

Steele, J.L., M.E. Johnson, J.R. Broadbent, and B.C. Weimer. 1998. Starter culture attributes which affect cheese flavor development, pp. 157-170. *In*, Proc. LACTIC '97 conference, Which strains? For which products?

Johnson, M.E., J.L. Steele, J. Broadbent, and B.C. Weimer. 1998. Manufacture of Gouda and flavor development in reduced-fat Cheddar cheese. Aust. J. Dairy Technol. 53:67-69.

Broadbent, J.R., M. Strickland, B. Weimer, M.E. Johnson, and J.L. Steele. 1998. Peptide accumulation and bitterness in Cheddar cheese made using

single-strain *Lactococcus lactis* starters with distinct proteinase specificities. J. Dairy Sci. 81:327-337.

Theses:

none

Published Abstract:

none

Presentations:

Broadbent, J.R. Role of lactic acid bacteria in cheese flavor development. Invited oral presentation for special ADSA pre-meeting workshop on the basics of flavor development in cheese. Sponsored by the American Dairy Science Association and Rhodia, Inc. July 24, Baltimore, MD.

Steele, J.L. 1999. Peptidases and amino acid catabolism. Invited oral presentation for the symposium on Dairy Flavors and Biotechnology. IFT Annual Meeting, July.

Steele, J.L. 1999. Peptidases and amino acid catabolism. Invited oral presentation for the Sixth Symposium on Lactic Acid Bacteria. September, The Netherlands.

Broadbent, J.R. 1999. Cheese curing and flavor development. Invited oral presentation for the 15th Cheese Making Short Course. February 9-11, Utah State University, Logan.

Broadbent, J.R. 1998. How starter bacteria direct cheese flavor development. Invited oral presentation for the 13th Biennial Cheese Conference. Aug. 10-12, Utah State University.

Broadbent, J.R. 1998. Influence of *Lactococcus lactis* starter bacteria on peptide accumulation and bitterness in Cheddar cheese. Invited oral presentation for Texel International. April 30, Dange, France.

Broadbent, J.R. 1998. Cheese curing and flavor development. Invited oral presentation for the 14th Cheese Making Short Course. March 26-28, Utah State University, Logan.

Broadbent, J.R. 1997. Influence of starter bacteria on peptide accumulation and bitterness in Cheddar cheese. Invited oral presentation for the National Cheese Technology Forum sponsored by Dairy Management, Inc., Dec. 9-10. Chicago.

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 — June 30, 2000

Principal Investigators: **Dr. Jeffery R. Broadbent, Utah State University**

Co-Investigators: Dr. Bart C. Weimer, Utah State University
Dr. James L. Steele, University of Wisconsin-Madison
Dr. Mark E. Johnson, Center for Dairy Research and
Dr. Scott A. Rankin, University of Maryland

Project Title: **Improvement of low-fat Cheddar 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 #: 97087

Project Completion Date: 12-31-00

National Research Plan (1997): Priority: 1; Goal: 1; Tactic: 2;
Clarify which organisms are responsible for cheese flavor (positive and negative) and understand how these organisms assert their influence.

Modifications to Project/Budget:
None

Project Objectives: (Include any revisions to objectives)

1. Investigate the role of metabolic cross-feeding between starter, adjunct, and nonstarter cheese bacteria in the production or removal of aromatic off flavor compounds.
2. Characterize the contribution of key enzymes to the conversion of aromatic amino acids into off flavor compounds.
3. Confirm the action of key enzymes in cheese slurries or in low-fat Cheddar cheese.

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

Development of off flavors is a significant problem in low-fat Cheddar cheese. Compounds associated with unclean, medicinal or utensil, and floral or rosy off flavors may arise via microbial catabolism of aromatic amino acids. Starter, adjunct, and nonstarter lactic acid 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% NaC, 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, The *L. casei* gene encoding the NADH-dependent D-2-hydroxyisocaproate dehydrogenase was cloned, sequenced, and overexpressed in *Lb. casei* 334 using the high copy number plasmid pTRKH2. Duplicate vats of 50% reduced fat Cheddar cheese were manufactured at UW-Madson using this strain and trained sensory evaluation was performed at UW. The volatile fraction of these cheese will now be analyzed at the University of MD to investigate the effect of DHic overexpression on the accumulation of cheese volatiles during ripening, and their possible relationship to sensory attributes.

2. Significant Conclusions:

Starter, 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 of flavor compounds.

3. Anticipated Problems/Delays:

None.

Publications:

Gummalla, S., and J.R. Broadbent. 1999. Tryptophan catabolism by *Lactobacillus casei* and *Lactobacillus helveticus* cheese flavor adjuncts. J. Dairy Sci. 82:2070-2077.

Steele, J.L., M.E. Johnson, J.R. Broadbent, and B.C. Weimer. 1998. Starter culture attributes which affect cheese flavor development, pp. 157-170. *In*, Proc. LACTIC '97 conference, Which strains? For which products?

Gao, S., D-H. Oh, J.R. Broadbent, M.E. Johnson, B.C. Weimer, and J.L. Steele. 1997. Aromatic amino acid catabolism by lactococci. Lait 77:371-381.

Broadbent, J., C. Brennand, M. Johnson, J. Steele, M. Strickland, and B. Weimer. 1997. Starter contribution to reduced fat Cheddar. Dairy Ind. Int. 62 (2):35-39.

Theses:

Gummalla, S. 1998. Tryptophan catabolism in *Lactobacillus* spp. MS thesis, Utah State University.

Published Abstract:

none

Presentations:

Broadbent, J.R. Role of lactic acid bacteria in cheese flavor development. Invited oral presentation for special ADSA pre-meeting workshop on the basics of flavor development in cheese. Sponsored by the American Dairy Science Association and Rhodia, Inc. July 24, Baltimore, MD.

Broadbent, J.R. and S. Gummalla. Tryptophan Catabolism by *Lactobacillus casei* and *Lactobacillus helveticus* cheese flavor adjuncts. Poster presentation at the 6th International Symposium on Lactic Acid Bacteria Genetics, Metabolism, and Applications. September 19-23, Veldhoven, The Netherlands.

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

Broadbent, J.R. 1998. How starter bacteria direct cheese flavor development. Invited oral presentation for the 13th Biennial Cheese Conference. Aug. 10-12, Utah State University.

Broadbent, J.R. 1998. Cheese curing and flavor development. Invited oral presentation for the 14th Cheese Making Short Course. March 26-28, Utah State University, Logan.

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 — June 30, 2000

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: 6-30-00

National Research Plan (1997): Priority: 1 Goal:1 Tactic: 1
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 inthe process of finding a new student or post doc to finish the project.

Publications:

Weimer, B. 1999. Brevibacteria. *In* Encyclopedia of Food Microbiology, R. K. Robinson et al. (Ed.), Academic Press, London.

Wahls, K. and **B. C. Weimer.** Diversity of sulfur compounds in lactic acid bacteria (submitted).

Umnadi, M. and **B. C. Weimer.** 1999. Use of capillary electrophoresis-laser induced fluorescence detection to monitor bacterial growth and amino acid utilization. Electrophoresis (accepted).

Dias,B., and **B. C. Weimer**. 1999. Detection of α–keto acids with capillary electrophoresis in culture supernatants and cheese. J Chrom A (accepted).

Dias,B., and **B. C. Weimer**. 1999. Production of volatile sulfur compounds in Cheddar cheese slurries. International Dairy Journal 9:605–611.

Weiner, B., K. Seefeldt, and B. Dias. 1999. Sulfur metabolism in bacteria associated with cheese. Antonie van Leeuwenhoek 76:247–261

Steele, J.L., M.E. Johnson, J.R. Broadbent, and **B.C. Weimer**. 1998. Starter culture attributes which affect cheese flavor development, pp. 157-170. *In*, Proc. LACTIC '97 conference, Which strains? For which products?

M. E Johnson, J. L. Steele, J. Broadbent, and B. C. Weimer. 1998. Manufacture of gouda and flavour development in reduced–fat cheddar cheese. Aust. J. Dairy Tech 53:67.

Dias B., and Bart Weimer. 1998. Conversion of methionine to thiols by lactococci, lactobacilli, and brevibacteria. Appl. Environ. Microbiol. 64:3320.

Dias B., and Bart Weimer. 1998. Purification and characterization of methionine γ–lyase from Brevibacterium linens BL2 Appl. Environ. Microbiol. 64:3327.

Broalbent, J.R., M. Strickland, B. Weimer, M.E. Johnson, and J.L. Steele. 1998. Peptde accumulation and bitterness in Cheddar cheese made using single-strain Lactococcus lactis starters with distinct proteinase specificities. J. Dairy Sci. 81:37.

Broalbent, Jeffery R., Charlotte Brennand, Mark E. Johnson, James L. Steele, Mare Strickland, and Bart C. Weimer. 1997. Contributions by starter and seleced adjunct bacteria to flavor development in reduced-fat cheddar cheese. Dairy Industry Int. 62:35.

Gao,S., D–H. Oh, J. Broadbent, M. Johnson, B. Weimer, and J. Steele. 1997. Aronatic amino acid catabolism by lactococci. Le Lait 77:371.

Weiner, B. C., C. Brennand, J. Broadbent, J. Jaegi, M. Johnson, F. Milani, J. Steele, and J. Sisson. 1997. Influence of flavor adjunct bacteria on the flavor and texture of 60% reduced fat Cheddar cheese. Le Lait 77:383.

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

Published Abstract:

———

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

Weimer, B. C., 1999. Production of flavor compounds by bacteria in cheese. University of California – Davis, Dept. of Food Science and Nutrition and Dept. of Enology.

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

Weimer, B. C., 1999. Sulfur metabolism in dairy related bacteria. IFT Biotechnology Section Keynote.

Weimer, B. C., 1999. Sulfur metabolism in bacteria associated with cheese. 6th FEMS International Conference, Keynote speaker. Veldhoven, The Netherlands.

Weimer, B. C., B. Dias, M. Ummadi, M. Stickland, J. Broadbent, M. Johnson, J. Jeaggi, J. Steele, and J. Harper. 1997. Improving Cheddar cheese flavor with the addition of brevibacteria. American Dairy Science Association Annual meeting.

Weimer, B. C., 1997. Strategies for improving cheese flavor. Dairy Managment, Inc. Conference on Cheese Flavor. Chicago, IL.

Patent/Invention Disclosures:

Technology Transfer Activities

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

Visitors Hosted:
Paul Chiak –IFF

Chakra Wijesundera – Food Science Australia

Western Dairy Center

Project Report

Reporting Period July 1, 1997 — June 30, 2000

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: 6-30-00

National Research Plan (1997): Priority: 1 Goal: 1 Tactic: 1
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, micrococci, 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:

Weimer, B. C., B. Ganesan, and K. Seefeldt. 2000. Volatile fatty acid production by starter and adjunct bacteria. International Dairy Federation Biennial Cheese Flavor Conference, Banff, Alberta.

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 January 1, 1997 — June 30, 2000

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**

Institution's 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 LH212 *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 cheese. 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. These 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 to 52C. 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 isolated to date do not display any GusA activity and DNA sequence analysis showed 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 constituitive *gusA* expression from the LH212 promoter. To overcome this problem, we have begun cloning experiments in *Lactococcus lactis*, where constitutive expression should not occur due to this bacterium's ability to recognize the heat shock negative regulatory elements (CIRCE) on the LH212 promoter.

Efforts are also underway to construct an expression vector that incorporates the LH212 *groESL* promoter upstream of the *Bacillus subtilis nprE* gene. This work was initially based on the broad host range, theta-replicating vector pHW800, but experiments showed this vector did not transform efficiently into *Lc. lactis*. As a result, we are now using a different vector for these studies.

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

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. These 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 to 52C. 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 isolated to date do not display any GusA activity and DNA sequence analysis showed 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 constituitive *gusA* expression from the LH212 promoter. To overcome this problem, we have begun cloning experiments in *Lactococcus lactis*, where constitutive expression should not occur due to this bacterium's ability to recognize the heat shock negative regulatory elements (CIRCE) on the LH212 promoter.

Efforts are also underway to construct an expression vector that incorporates the LH212 *groESL* promoter upstream of the *Bacillus subtilis nprE* gene. This work was initially based on the broad host range, theta-replicating vector pHW800, but experiments showed this vector did not transform efficiently into *Lc. lactis*. As a result, we are now using a different vector for these studies.

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

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 by the manufacturer. 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 reaction and sugar utilization (API), and are currently being evaluated by membrane fatty acid analysis. Ten additional commercial strains of lactobacilli have been received and characterized.

Objective 1, Step 2 - Sixteen strains of lactobacilli (7 strains of *Lactobacillus helveticus* and 9 strains of *Lactobacillus delbruekii* ssp. *bulgaricus*) have been characterized for proteolysis using the α_{s1}-casein (f 1-23) method. Analysis for the remaining ten commercial strains is currently underway. HPLC analysis of whole cell preparations of nine different *L. delbrueckii* subsp. *bulgaricus* and six distinct *L. helveticus* strains incubated with a_{s1}-casein (f 1-23) detected at least six proteolytic patterns. Clustering of these patterns within each species was apparent, but overlaps were also noted. All cells accumulated a_{s1}-casein (f 1-9), but differences were found in both the primary and secondary specificity toward a_{s1}-casein (f 1-23) and its breakdown products. In addition, while some strains exhibited an identical specificity, they showed a much higher or lower relative affinity toward individual peptide bonds.

Objective 1, Step 4 - OPA analysis for total proteolysis has been done for the characterized strains. The *o*-phthaldialdehyde (OPA) method was used to determine general proteolysis for each bacterial strain. No correlation was found between OPA data and with a_{s1}-casein (f 1-23) cleavage profiles.

Proteolysis in Mozzarella cheese by bacterial starter cultures influences cheese functional properties, but the molecular basis for these observations remains relatively obscure. Differences in the proteolytic activity of individual cultures has also been linked to variability in Mozzarella cheese melt and stretch properties. For this reason, we investigated the diversity in specificity of cell-bound extracellular proteinases in the Mozzarella starter cultures *Lactobacillus helveticus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*.

2. Significant Conclusions:

Preliminary data for the α_{s1}-casein (f 1-23) method indicates six distinct types of proteolysis can be found in dairy lactobacilli. Differences in preferential cleavage patterns of the α_{s1}-casein (f 1-23) for various strains the can be used for characterization and cleavage patterns for unique strains are being developed. An improved understanding of the influence of *Lactobacillus* proteinase specificity on casein degradation and cheese functionality should facilitate efforts to develop starter cultures that improve the physical and functional properties of Mozzarella cheese.

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, particularly from commercial sources. No other delays are anticipated.

Publications:

Oberg, C. J., J. R. Broadbent, M. Strickland, and D. J. McMahon. 2000. Diversity in specificity of the extracellular proteinases in *Lactobacillus helveticus* and *Lactobacillus delbruecki* subsp. *bulgaricus*. Letters in Applied Microbiology (in review).

Theses:

None

Published Abstract:

Oberg, C. J., J. R. Broadbent, M. Strickland, and D. J. McMahon. 2000. Diversity in specificity of the extracellular proteinases in *Lactobacillus helveticus* and *Lactobacillus delbruecki* subsp. *bulgaricus*. ASM Annual Meeting Program.

Presentations:

Oberg, C. J., J. R. Broadbent, M. Strickland, and D. J. McMahon. 2000. Diversity in specificity of the extracellular proteinases in *Lactobacillus helveticus* and *Lactobacillus delbruecki* subsp. *bulgaricus*. ASM Annual meeting. Los Angeles, CA. May 22-25, 2000.

Patent/Invention Disclosures:

Technology Transfer Activities

For information on licensing contact:

Western Dairy Center

Project Report

Reporting Period January 1, 1998 — June 30, 2000

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 #: 98093

Project Completion Date: December 31, 2000

National Cheese Research Plan (1997): Priority: 2 **Goal:** 2 **Tactic:** 1
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 1x 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, but

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:

Dave, R. I., McMahon, D. J., Oberg, C. J, and J. R. Broadbent. 1999. Influence of coagulant concentration on proteolysis, meltability and rheology of 0, 10 and 20% fat containing Mozzarella cheese made using direct acidification. 94th American Dairy Science Association Meeting, J. Dairy Sci. 82(Supp. 1):D59.

Presentations:

Dave, R. I., McMahon, D. J., Oberg, C. J, and J. R. Broadbent. 1999. Influence of coagulant concentration on proteolysis, meltability and rheology of 0, 10 and 20% fat containing Mozzarella cheese made using direct acidification. 94th American Dairy Science Association Meeting, J. Dairy Sci. 82(Supp. 1):D59.

Patent/Invention Disclosures:

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

Visitors Hosted:

Western Dairy Center

Project Report

Reporting Period January 1, 1998 — June 30, 2000

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

National Milk/ Whey Powders Research Plan (1997): Priority: Goal: 2 **Tactic:**2
Increase the use of whey and whey products for health and nutrition applications/ positionings (include carries for nutraceuticals, pharmaceuticals, vitamins, essential nutrents).

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 2% whey solution, the matrix was washed with sodium acetate buffer at pH 4 containing1 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-TFF 2.5 ft^2 cartridge membrane with 10 kDa (Millipore, Bedford, MA) to remove lactose. The efficiency of lactose removal was confirmed with a lactose enzymatic bioanalysis kit (Boehringer Mannheim, Indianapolis, IN). Moisture, protein, minerals and lipid content of lactose free buttermilk were determined by oven, Kjeldahl, ash and Majonnier, respectively. Lactose free buttermilk was freeze-dried and gangliosides were extracted using 20 vol. of organic mixture of an 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 Jourdian et al. (9).

Ganglioside Immobilization

Controlled pore glass (CPG) beads (2000 A°, 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 lyso-derivative containing a free amino group by the reflux boiling method (22). The OPA method (O-phthaldialdehyde) as described by Weimer and Oberg (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), 1 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 containing1 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 isoelectrofocusing.

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:

Rapid Fractionation of Bovine transferrin using immobilized gangliosides. M.K. Walsh and S.H. Nam. In Press for Preparative Biochemistry and Biotechnology.

Affinity Purification of Bovine Lactoferrin from Whey. M.K. Walsh and S.H. Nam. In press for In Press for Preparative Biochemistry and Biotechnology.

Theses:

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

Published Abstracts/ Presentations:

S.E. Nam and M.K. Walsh. Affinity purification of prosaposin from whey. Institute of Food Technologists, June 1998.

S.E. Nam and M.K. Walsh. 1999. Affinity purification of bovine lactoferrin from whey.

Institute of Food Technologists Annual Meeting, Chicago, IL.

S.E. Nam and M.K. Walsh. 2000. Affinity purification of transferrin from whey. ADSA Annual Meeting, Baltimore MD.

Patent/Invention Disclosures:

Technology Transfer Activities
For information on licensing contact:
Provisional patent submitted

Visitors Hosted:

Scott Bloomer, Land O'Lakes, Inc.

Western Dairy Center Report Addendum
December 31, 1997

Confidential - For Center Director's Use Only
(Release of this information requires prior approval by principal investigator)

Provide summary of significant advances in project that you wish to remain confidential at this time. For example, new methodology that you do not with to release until the current work is completed; information on invention disclosures or patents filed; activities conducted to transfer the technology to industry, or licensing agreements that are underway.

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

Invention Disclosures: (Title, Date)

_____

Patents: (Title, Date, #)

_____

Licensing Activities:

_____

Discoveries:

_____

Western Dairy Center

Project Report

Reporting Period January 1, 1998 — June 30, 2000

Principal Investigators: 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

National Milk/Whey Powders Research Plan (1997): Priority: Goal: 4 **Tactic:**1
Develop new applications for whey, NDM and their components.

Modifications to Project/Budget:
None

Project Objectives: (Include any revisions to objectives)

We believe that thermoplastic 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 textured 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 in hamburger patties. Representative TWPs were chosen for the first panel based on water holding capacity. The highest scoring TWP from the first panel was tested in a second panel at three usage levels, 30, 40 and 50%.

Panel one

In the first panel, 83 panelists tested six samples including: 1) TWP extruded with water, 2) TWP extruded with 0.1 M HCl, 3) TWP extruded with 0.2 M NaOH, 4) TWP extruded with water with 1.69% calcium (w/w protein) added, 5) a textured soy protein meat extender, and 6) an all beef control. The objective was to compare the acceptability of TWPs to commonly used products. All meat extenders were added to the ground beef at the 30% level (weight hydrated/weight patty). All patties were standardized to 20% fat.

Panel 2

In panel two, three usage levels of the most acceptable TWP from panel 1,TWP extruded with 0.2 M NaOH, were tested, 30, 40 and 50%, and compared to an all beef control. Salt was added to each meat mix 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 were served hot samples in booths, under red lights, in an open 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). 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 for flavor.

The 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 effect 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 the 40% level. This research shows high usage potential for textured whey proteins as meat extenders.

3. Anticipated Problems/Delays:

None

Publications:

_____

Theses:

Effects of pH and calcium level on extrusion-textured whey protein products. A.B. Hale. Utah State University.

Published Abstract:

Temperature, pH and calcium effects on the protein solubility of an extruded whey protein product. A. B. Hale, B. C. Pettee, C. E. Carpenter and M. K. Walsh. 1999 ADSA Annual Meeting, Memphis, TN.

Acceptable usage levels of textured whey proteins in hamburger patties. A.B. Hale, C. Carpenter, and M.K. Walsh. ADSA/ASAS Meeting, Baltimore MD, June 2000.

Sensory analysis of textured whey protein meat extender in hamburger patties. M.K. Walsh, A.B. Hale and C. Carpenter. IFT Annual Meeting, Dallas TX, July, 2000.

Presentations:

Same as above

Patent/Invention Disclosures:

Provisional and Patent pending

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

Visitors Hosted:

Western Dairy Center Report Addendum
December 31, 1997

Confidential - For Center Director's Use Only
(Release of this information requires prior approval by principal investigator)

Provide summary of significant advances in project that you wish to remain confidential at this time. For example, new methodology that you do not with to release until the current work is completed; information on invention disclosures or patents filed; activities conducted to transfer the technology to industry,or licensing agreements that are underway.

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

Invention Disclosures: (Title, Date)

_____

Patents: (Title, Date, #)

_____

Licensing Activities:

_____

Discoveries:

_____

Western Dairy Center

Project Report

Reporting Period January 1, 1998 — June 30, 2000

Principal Investigators: **Deborah R. Gustafson**
Co-Investigators:

Project Title: **Appetite Suppressing Properties of a Peptide from Milk**

Institution's Project #: 89098

Project Completion Date: Summer 2000

National Research Plan (1997): Priority: milk/whey powder **Goal:** 2 **Tactic:** 2
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. If so, 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 no- 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 the 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 colorant and clouding agent. Subjects were randomized into the study using a Latin Square randomization. Treatment beverages and ad libitum lunches were consumed on four separate occasions at the Study Center. Beverages were consumed one hour prior to lunch. After lunch, subjects were free to leave, but completed a short standardized questionnaire every hour throughout the afternoon and evening to assess feelings of hunger and

stomach fullness. Subjects also kept track of all food and beverages consumed away from the Study 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 effect on subjective indicators of satiety.
Conclusions: Intake of CMP before a midday meal has no effect on regulation of food intake over a short-term period.

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

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

Theses:

Published Abstract/Presentation:
"Appetite Suppressing Properties of a Peptide from Milk?". Poster presentation at Nutrition Week 1999, sponsored by the International Dairy Federation and National Dairy Council, October 4-7, 1999, in San Francisco

Presentations:

Patent/Invention Disclosures:

Technology Transfer Activities For information on licensing contact:

Visitors Hosted:

Western Dairy Center Report Addendum
December 31, 1997

Confidential - For Center Director's Use Only
(Release of this information requires prior approval by principal investigator)

Provide summary of significant advances in project that you wish to remain confidential at this time. For example, new methodology that you do not with to release until the current work is completed; information on invention disclosures or patents filed; activities conducted to transfer the technology to industry, or licensing agreements that are underway.

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

Invention Disclosures: (Title, Date)

Patents: (Title, Date, #)

Licensing Activities:

Discoveries:

Western Dairy Center

Project Report

Reporting Period January 1, 1998 — June 30, 2000

Principal Investigators: **Joseph Irudayaraj, Penn State University**
Co-Investigators: Donald McMahon__

Project Title: **Process technology to improve the favor of heated milk**

Institution's Project #: 98101

Project Completion Date: June 30, 2000

National Fluid Milk Research Plan (1997): Priority: 3 **Goal:** 3 **Tactic:**
Fluid Milk 3.3 : Exploration of alternate processes for specific products and development of ingredients and beverages____

Modifications to Project/Budget:

_____

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

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. Another set of experiment was conducted using the commercial pasteurized milk. Electroheating temperatures considered were 135, 145, and 155 deg C. Holding times used were 0.5 and 4 secs. All experiments were replicated two times.

Protein denaturation from electroheating was 30% while that of the commercial UHT sample was about 67%. Sensory evaluation using a trained (12 panelists) and untrained panel (80 consumers) was conducted. The commercial sample had the lowest liking scores from the untrained panelists and 66% of the consumers preferred the UHT milk by electroheating (145 deg C) over the commercial variety. High sweetness, low butter, oxidized, and stale flavor was realized by the trained panel for the electroheated milk.

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 "Du-pont 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 that 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 heaing 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:

$$\% \text{ Denaturation} = 100 * (WPN_{\text{raw milk}} - WPN_{\text{heated milk}}) / WPN_{\text{raw milk}}$$

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 1C 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 40C isothermic for 6 min; increased by 6C/min to 210C and maintained for 15 min. A Restek (Bellefonte, PA) Stabilwax-DA column is employed (30 m, 0.25 mm i.d., 1.0 l film thickness). Column flow rate is 1.1 ml/min and sample size is 1.5 l 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, 170C; 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 1 : Protein denaturation of milk

Sr. No.	Sample ID	Trial 1 % Denaturation	Trial 2 % Denaturation	Trial 3 % Denaturation
1	Past. Milk 1	0.0	0.0	0.0
2	GS 2% fat	70.54	66.17	66.17
3	GS Skim milk	70.54	-	-
4	Treatment1 (155, 1 sec)	23.65	28.65	28.91
5	Treatment2 (145, 1 sec)	21.04	21.1	21.11

6	Treatment3 (145, 4 sec)	35.26	36.95	36.95
7	Treatment4 (135, 4 sec)	27.35	30.21	30.18

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[1] for flavor attributes of 2 % UHT milk

Treatment		Sweet	Sour	Salt	Bitter	Dairy	Cooked	Oxidized	
	0.38[2]	0.24	0.27	0.11	0.64	0.91	0.67	0.65	0.42
Control	0.9[B3]	0.3[A]	0.4[A]	0.2[A]	5.5[A]	1.6[A]	1.8[A]	1.4[A]	1.2[B]
Treatment 1	1.6[A]	0.3[A]	0.3[A]	0.1[A]	6.0[A]	1.9[A]	1.3[AB]	0.7[AB]	0.4[A]
Treatment 2	1.6[A]		0.3[A]	0.4[A]	0.1[A]	5.8[A]	1.9[A]	1.1[B]	0.6[B]
Treatment 3	1.6[A]	0.3[A]	0.4[A]	0.1[A]	6.1[A]	2.4[A]	1.3[AB]	1.0[AB]	0.5[A]
Treatment 4	1.5[A]		0.2[A]	0.5[A]	0.1[A]	5.8[A]	2.4[A]	1.3[AB]	0.7[AI]

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

Table 3. Mean overall liking scores[1] of UHT milk

Treatment	Overall Liking[2]
[3]	0.69
Control	4.2[B4]
Treatment 1	5.7[A]
Treatment 2	6.1[A]
Treatment 3	5.6[A]
Treatment 4	5.6[A]

[1] n=79 (40 persons evaluated rep1 samples; 39 persons evaluated rep 2 samples)
[2]Minimum significant difference
[2]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
[3]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

	% responding to each Category				
Question	Control	Trt 1	Trt 2	Trt 3	Trt 4
In comparison to milk you normally Drink, the milk sample was :					
Better than	13.9	13.9	15.1	13.9	13.9
Equal to	13.9	37.9	51.8	40.5	45.5
Worse than	72.1	48.1	32.9	45.5	40.5

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

Treatment	Sweet	Sour	Salt	Bitter	Dairy	Cooked	Oxidized	
	0.38[2]0.24	0.22	0.09	0.61	0.82	0.87	0.51	0.40
Control	1.0[B3] 0.4[A]	0.3[A]	0.2[A]	6.2[A]	2.1[A]	2.0[A]	0.8[A]	1.3[A]
Treatment 1	1.6[A]	0.4[A]	0.4[A]	0.1[A]	6.0[A]	2.4[A]	1.4[AB]	0.4[A] 0.7[B]
Treatment 2	1.4[A]	0.3[A]	0.4[A]	0.1[A]	6.1[A]	1.9[A]	1.1[B]	0.3[A] 0.6[B]
Treatment 3	1.2[AB]	0.3[A]	0.3[A]	0.1[A]	6.2[A]	1.8[A]	1.1[B]	0.4[A] 0.8[B]
Treatment 4	1.3[AB]	0.3[A]	0.4[A]	0.2[A]	6.3[A]	1.7[A]	1.3[AB]	0.6[A] 0.6[B]

[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

Treatment	Overall Liking[2]
3	0.71[2]
Control	3.8[B4]
Treatment 1	5.9[A]

Treatment 2	6.0[A]
Treatment 3	6.0[A]
Treatment 4	6.2[A]

[1] n = 79 (40 persons evaluated rep 1 samples; 39 persons evaluated rep 2 samples)
[2] Minimum significant difference.
[2] 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.
[3] 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"

Percent responding to each category

Question	Control	Trt 1	Trt 2	Trt 3	Trt 4
Better than	3.8%	13.9%	12.7%	16.5%	15.2%
Equal to	16.5%	40.5%	46.8%	41.8%	57.0%
Worse than	79.7%	45.6%	40.5%	41.8%	27.8%

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

Brewer, C.E., Tyszkiewicz, R.B., and Rankin, S.A. 1997. Presented at American Dairy Science Association annual meeting, Guelph, ON, Canada, June 1997.

Moio, L., Dekimpe, J., Etievant, P.X., and Addeo, F. 1993. Ital. J. Food Sci. 5: 57-68.

3. Anticipated Problems/Delays:

Due to the tremendous volume of work a minor delay in GC analysis was anticipated. However, this was rectified.

Publications:

___none__

Theses:

__none___

Published Abstract:

__none___

Presentations:

___none__

Patent/Invention Disclosures:

Technology Transfer Activities
For information on licensing contact:
_____

Visitors Hosted:

___none__

Western Dairy Center

Project Report

Reporting Period January 1, 2000 - June 30, 2000

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 #: 99104

Project Completion Date: 12/31/01

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

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)

Progress

Effect of water and calcium injection on structure-function attributes of Mozzarella cheese

Introduction

In cheese, calcium content is associated with differences in structure-function attributes. However, the specific way in which calcium influences such structure-function attributes of cheese requires further elucidation. Our objective was to determine how injection of calcium solutions into cheese affects cheese microstructure, and then relate that to changes in hardness and melting of cheese.

Materials and Methods

Mozzarella cheese (48, 49, and 53% moisture, and 22% fat) was made by a direct-acid, stirred-curd procedure. Cheese was cut into 0.3 to 0.4-kg blocks, vacuum packaged and stored for 10 d at 4°C. Cheese blocks were then high-pressure injected (1 to 5 times) with either water or a 40% calcium chloride solution. Thus, ten treatments were defined, corresponding to five water and five calcium injection levels. A control, uninjected cheese block was also considered. Injections were performed in two opposite sides of the block, and according to a 1 x 1 cm pattern, with successive injections performed 24 h apart. Pressure of injection was set as 1400 psig, and burst injection time as 1 s. After 42 d of storage at 4°C, cheese blocks were analyzed for structural and functional attributes. Scanning electron micrographs, (1500 X magnification, from two different fields) were uploaded into Adobe Photoshop® 4.0 and their gray-scale values analyzed. Dark areas (corresponding to fat/whey pockets) were differentiated from light areas (corresponding to protein matrix) by applying a threshold function, and the proportion of pixels associated with dark and light areas determined. Texture profile analysis was performed using a two-bite compression test run on an Instron 5542 (Canton, MA). Samples, 20 mm by 16 mm diameter, were taken from the cheese immediately after removal from the refrigerator and tested at ~5°C.

Results and Discussion

Results of the statistical analysis are presented in Table 1. When water was injected, a slight increase in weight was observed. In contrast, when calcium was injected, the cheese lost weight and considerable serum was expelled from the cheese. Moisture content increased with water injection, and decreased with calcium injection. The control (uninjected) cheese had the typical structure of a stirred/pressed-curd cheese, with protein matrix interspersed with areas containing fat and/or serum. Injecting water increased the area occupied by the protein matrix (by 14% after 5 injections) as shown by an increase in the proportion of light pixels in the micrographs. Increasing the calcium content of the cheese (from 0.3% to 1.8% after 5 injections) decreased the area occupied by the protein matrix (by 17%). This represents a contraction of the

protein matrix and concomitant release of serum entrapped within the protein matrix. A decrease in cheese pH occurred upon injection of calcium, but it had been previously observed that pH did not affect cheese microstructure unless it was accompanied by a change in calcium content. Water injection decreased cheese hardness but did not affect any other functional attribute. Hardness increased when calcium was injected, but cohesiveness decreased. Adhesiveness and springiness were unaffected. Meltability of the cheese was inversely proportional to calcium content.

Table 1. Pr>F for the ANOVA sources of variation and specified contrasts by variable of interest.

Variable	Calcium	Moisture	pH	Weight	Melting	Hardness
Source						
Model	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Block	< 0.0001	< 0.0001	< 0.0001	0.0634	0.0005	< 0.0001
Treatment	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Determination	0.6707	0.9705	0.8773		0.9841	0.7387
Contrast						
Control-Calcium[1]	< 0.0001	< 0.0001	< 0.0001	< 0.0001	<0.0001	< 0.0001
Control-Water[2]	0.6225	0.0253	0.0105	0.1418	0.9455	0.0254

[1]. Contrast of control (uninjected cheese) against all calcium levels.
[2]. Contrast of control (uninjected cheese) against all water levels.

Conclusions

Increasing calcium content of cheese alters how proteins in the cheese matrix interact. It appears that calcium promotes protein-to-protein interactions, probably through calcium bridging and charge neutralization. Such increased interactions between proteins cause contraction of the protein matrix and expulsion of serum from the matrix. More energy must also be applied to overcome these interactions and allow

proteins to flow when heated. Thus, cheese hardness is increased and meltability decreased when the calcium content of the cheese is increased.

Presentations

- 04/2000. Utah Academy of Sciences, Arts & Letters. Southern Utah University. Cedar City, UT.

- 06/2000. Institute of Food Technologists. 2000 Annual Meeting. Dallas, TX.

Published Abstract

A.J. Pastorino, N.P. Ricks, C. Hansen and D.J. McMahon. 2000. Effect of water and calcium injection on structure-function attributes of Mozzarella cheese. Institute of Food Technologists. 2000 Annual Meeting & FOOD EXPO. Dallas, TX.

Western Dairy Center

Project Report

Reporting Period July 1, 1999 - June 30, 2000

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 #: 99106

Project Completion Date: 2/01/2001

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**:

Since our last update, we have made significant progress in the completion of this project. Most importantly, upon gaining the approval of the Institutional Review Board, we began subject recruitment in June. Since that time, we have successfully enrolled all 12 women, and have completed sample and data collection in 10 of them. The remaining 2 subjects will complete the study in early September. In summary, this experiment lasts 32 d and has 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 consists of enriching the diets of the women with butter (containing CLA and no industrially-produced trans fatty acids), regular margarine (containing no CLA and high amounts of industrially-produced trans fatty acids) and no trans margarine (containing either CLA nor industrially-produced trans fatty acids). Blood and milk samples are collected 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; DEXA) during the baseline period.

Milk and plasma fat analyses will commence in early September, with fatty analyses following this. It is our hope that milk fat data will be submitted in

abstract form for the upcoming Experimental Biology meetings (Spring, 2001). Dietary data entry into computerized dietary assessment software is currently underway; we hope to complete this by November. It is our hope that all laboratory analyses will be completed by May, 2001 with statistical analyses and manuscript preparation completed by August, 2001. We anticipate the publication of one peer-reviewed journal article from this research.

2. **Significant Conclusions**:

None to date.

3. **Anticipated Problems/Delays**:

None to date.

Publications:

None to date.

Theses:

None to date.

Published Abstract:

None to date.

Presentations:

None to date.

Patent/Invention Disclosures:

None to date.

Technology Transfer Activities

None to date.

Visitors Hosted:

None to date.

Western Dairy Center Report Addendum

Confidential - For Center Directorís Use Only

(Release of this information requires prior approval by principal investigator)

Provide summary of significant advances in project that you wish to remain confidential at this time. For example, new methodology that you do not with to release until the current work is completed; information on invention disclosures or patents filed; activities conducted to transfer the technology to industry, or licensing agreements that are underway.

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
Schedule for data collection

Western Dairy Center

Project Report

Reporting Period October 1, 1999 — June 30, 2000

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

Project Title: **Effect of size, hydrophobicity and temperature on the diffusion of molecules within the Cheddar cheese matrix.**

Institution's Project #: 99110

Project Completion Date: 4/1/2000

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

Modifications to Project/Budget:

Project Objectives: (Include any revisions to objectives)

1. Determine the extent of diffusion for macro molecules in Cheddar cheese.
2. If significant diffusion is detected in objective 1, determine the effect of storage temperature on the migration of molecules in Cheddar cheese.
3. Id significant diffusion is detected in objective 1, determine the effect of age at injection on the migration of molecules in Cheddar cheese.

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

1. Significant Progress against Objectives:

This project was delayed in starting because of problems with the confocal microscope and problems with personnel. We now have a person trained on the microscope and have determined the sample preparation procedures. We are currently collecting and analyzing images from the microscope.

2. Significant Conclusions:

3. Anticipated Problems/Delays:

We anticipate is project will bc completed by April, 2001.

Publications:

Theses:

Published Abstract:

Presentations:

Patent/Invention Disclosures:

Technology Transfer Activities

Visitors Hosted:

Western Dairy Center Report Addendum

Confidential - For Center Director's Use Only
(Release of this information requires prior approval by principal investigator)

Provide summary of significant advances in project that you wish to remain confidential at this time. For example, new methodology that you do not with to release until the current work is completed; information on invention disclosures or patents filed; activities conducted to transfer the technology to industry, or licensing agreements that are underway.

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

Invention Disclosures: (Title, Date)

Patents: (Title, Date, #)

Licensing Activities:

Discoveries:

Western Dairy Center

Project Report

Reporting Period January 1, 2000 — June 30, 2000

Principal Investigators: Marie Walsh
Co-Investigators:

Project Title: Production of a 100% textured whey product

Institution's Project #: 00112

Project Completion Date: 6/30/00

National Research Plan (1997): Priority: milk/whey powder **Goal:** 2 **Tactic:** 2

Modifications to Project/Budget:

Project Objectives: (Include any revisions to objectives)

The objective of this research was to evaluate a TWP pattie for consumer acceptance. A TWP was produced by thermoplastic extrusion. Binding agents for formulating meatless TWP burger patties were evaluated. Flavored TWP patties were compared to a comparably flavored commercially available TVP product using sensory analysis.

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

Whey protein concentrate (WPC, 80% protein) was textured using thermoplastic extrusion to produce textured whey protein (TWP) product. Various binding agents were investigated for their usefulness in the formation of a TWP meatless burger patty. Sensory analysis of two TWP patty formulations and a commercially available textured vegetable protein (TVP) patty was performed. Patties were evaluated on appearance, texture, flavor, aftertaste and overall acceptance. There were no significant differences among the appearance and texture of the three patties, whereas the flavor, aftertaste and overall acceptance were significantly different. The preference rankings for the TWP patties were significantly higher than the TVP patty.

1. Significant Progress against Objectives:

Pattie Formulation

Commercial meatless burger patties available at local supermarkets were sampled to evaluate the effectiveness of various binding agents in similar meatless patty products. The binding agents are incorporated in the formulation to hold the patty matrix together. Binding agents present in commercial meatless patties include brown rice, carob bean gum, corn oil, cornstarch, dried eggs, dried egg whites, gum arabic, konjac flour maltodextrin, potato flakes, safflower

oil, tapioca starch, wheat gluten, vegetable gum, and xanthan gum. The binding agents are most commonly used in combination to set and hold the meatless patty matrix during each processing step prior to consumption. In whey burger formulation these steps include rehydration of textured whey protein, patty formation, baking, freezing, thawing and reheating. It was necessary to determine which combination of binding agents, and in what concentration, resulted in the desired cohesiveness, texture, and mouthfeel in the textured whey burger patty.

From initial trials using binding agents in the patty formulation, it was determined that dehydrated whole eggs should be eliminated due to the predomination an off flavor (egg or sulfury), undesirable color (yellow hue), and changes in mouthfeel (coating of the mouth). Brown rice was not found to be an effective binding component in the textured whey burgers. It had no observable effect on the stability of the TWP matrix. Excessive levels of xanthan gum resulted in a slimy mouthfeel. Wheat gluten effectively bound the rehydrated textured whey protein fragments but when used in large amounts, undesirable glue-like masses were observed pooled in clumps as part of the stringy elastic gluten matrix. Egg white, gluten, and xanthan gum, especially when used in combination, proved to be very effective at binding the rehydrated textured whey protein fragments.

Differences in texture were identified in comparing the individual patties before and after freeze-thaw and reheating. During the freeze-thaw evaluation it became evident that although the burgers formulated with a larger amount of dehydrated egg whites held together well, they exhibited a distinct an undesirable grainy/sandy mouthfeel.

Additional patties were formulated using lower concentrations of dehydrated egg white to avoid the grainy/sandy defect while relying more on wheat gluten to provide patty cohesiveness. The formulation was optimized to reduce the use of wheat gluten, dehydrated egg whites, and xanthan gum. These binding agents were used at minimal levels while still offering the desired texture and binding. The final binding combination was determined to be 2.25 g egg white, 2 g wheat gluten, and 0.5 g xanthan gum per 30 g of textured whey protein particles rehydrated in 45 g of water (Table 1). As determined by the 4-member taste panel, this combination eliminated the undesirable grainy/sandy defect by reducing the amount of dehydrated egg white three fold. The small amount of xanthan gum did not result in the slimy mouthfeel defect but exhibited desirable uniform water binding. The level of gluten resulted in an appropriate level of stringiness without pooling the gluten into undesirable masses. TWP burger formulations used in the sensory evaluation are shown in Table 1.

Sensory

Mushroom and Vegetable flavored TWP burger patties were evaluated side-by-side in a preference test of three different samples. The third sample was a commercial product produced by GardenBurger® Inc. (GardenBurger® Inc., Portland, OR). The commercial product was Gardenburger®'s Hamburger Style Roasted Garlic Gardernburger® Soy Burgers ©1999 Gardenburger, Inc.)

Analysis of the results for the different attributes elucidates the comparison of the three products evaluated. Statistical Analysis Software (SAS) was used to generate analysis of variance tables for each of the five attributes scored in the sensory evaluation. The assumptions of approximate normality and equality of variance of the residuals were satisfied. Overall acceptability, aftertaste, and flavor were found to be highly statistically significant ($p<0.0001$ each). These attributes were also the main contributors to the explained variability (r-squared values of 0.1270, 0.1458, 0.1908 respectively). Appearance and texture were found to be non-significant ($p=0.7661$ and $p=0.0717$ respectively). These attributes did not contribute to the explained variability (r-squared values of 0.0022 and 0.0217 respectively). Texture was determined to be marginally non-significant in this evaluation. However, if the number of panelists increases in future testing, this attribute may become significant. The attribute effects are summarized in Table 2.

The mean scores, rank sums, and difference rankings as reported by SAS, including the multiple means comparison tests for the individual samples, are shown in Table 3. The two formulations of TWP patties scored significantly higher ($p<0.05$) than Gardenburger®'s Hamburger Style Roasted Garlic Gardernburger® Soy Burgers (©1999 Gardenburger, Inc.) in overall acceptability, aftertaste, and flavor. There was no significant difference ($p>0.05$) between the three products in appearance or texture. TWP patties were scored significantly higher ($p<0.05$) than Gardenburger®'s Hamburger Style Roasted Garlic Gardernburger® Soy Burgers (©1999 Gardenburger, Inc.) in preference ranking. The two formulations of TWP patties did not differ significantly from each other in any of the individual attributes or the preference ranking.

The demographics of the panelists were considered. Of the 81 volunteer panelists, 58% of the evaluators were female (47 panelists) and 42% male (34 panelists). The panelists were stratified into four age categories (15 to 24, 25 to 34, 35 to 45, 45 and above). Forty-three percent of those participating in the evaluation were ages 15 to 24 (35 panelists). Twenty-five percent were ages 25 to 34 (20 panelists). Twenty-two percent were ages 45 or more (18 panelists). Ten percent were ages 35 to 44 (8 panelists).

The panelists were asked to indicate how often they consumed meatless burger patties. The majority (75%) of the panelists consumed 0 to 1 meatless burger patties per month (64 of 81 panelists). Ten percent consumed 2 to 5 meatless burger patties per month (8 of 81 panelists). Five percent stated that they consumed 5 to 10 meatless burger patties and 5% that they consumed 10 or more meatless burger patties per month. One panelist did not indicate frequency of consumption.

Half the panelists (5 of 10) who frequently consume meatless burgers (5 or more per month) ranked Whey burgers above Gardenburger® Hamburger Style Roasted Garlic Gardenburger® Soy Burgers in the preference ranking portion of the evaluation. The majority (8 of 10) of these panelists who frequently consume meatless burgers (5 or more per month) rated one of the Whey burgers at least a point higher than Gardenburger® Hamburger Style Roasted Garlic Gardenburger® Soy Burgers in the overall acceptability portion of the evaluation.

This research determined how WPC, textured by thermoplastic extrusion, could be used in the formulation of meatless patties. Such formulae take

advantage of whey's mild flavor and complete protein nutritional profile in developing a product more desirable than a commercially available TVP meatless patty. Challenges identified extruding whey protein in similar applications prior to this investigation had to be overcome.

TABLE 1

TWP Burger Patty Formulations

Ingredient	Vegetable TWP (g)	Mushroom TWP (g)	Desired Effect
Textured Whey Protein	30	30	Mild dairy flavor, Texture
Water	45	45	Hydrate TWP, Juiciness
Corn Oil	3	3	Flavor, Processing Aid
Egg White (dry)	2.25	2.25	Binding of TWP, Texture
Wheat Gluten (dry)	2	2	Binding of TWP, Texture
Xanthan Gum (dry)	0.5	0.5	Binding of TWP, Texture
Vegetable Base	3	0	Flavor
Mushroom (raw)	0	6	Flavor, Texture
Mushroom Base (paste)	0	3	Flavor

TABLE 2

Analysis of variance models for sensory properties of Gardenburger® soy and TWP meatless patties

Attribute	Model	R-Squared	F Value	P-Value
Appearance	Not Significant	0.0022	0.27	0.7661
Texture	Non-Significant	0.0217	2.66	0.0717
Flavor	Significant	0.1908	28.30	<.0001
Aftertaste	Significant	0.1458	19.89	<0.001
Overall Acceptability	Significant	0.1270	17.46	<0.001

TABLE 3

Mean Scores, Rank Sums, and Difference Rankings

Description	A	B	C
Product	Garden Burger Garlic	TWP Mushroom	TWP Vegetable
Blinding Code	**258**	**314**	**769**

Appearance	5.20[a]	5.31[a]	5.42[a]	**NS** cr = 0.60
Texture	4.35[a]	4.85[a]	5.01[a]	**NS** cr = 0.59
Flavor	2.95[b]	5.11[a]	4.60[a]	**BC > A** cr = 0.59
Aftertaste	3.24[b]	4.89[a]	4.71[a]	**BC > A** cr = 0.57
Overall Acceptance	3.38[b]	5.00[a]	4.73[a]	**BC > A** cr = 0.58
Preference Ranking	121[b]	193[a]	172[a]	**BC>A** cd= 29.8

Like superscripts on means within a row indicate no significant difference among the means ($\alpha = 0.05$).
NS = not significantly different
cr = least significant difference critical range. Acceptability means differing by the cr or more are different ($\alpha = 0.05$).
cd = critical difference. Rank sums differing by more than the critical difference are different ($\alpha = 0.05$).

2. Significant Conclusions:

Whey protein concentrate (WPC) can be textured with cornstarch using thermoplastic extrusion to produce textured whey protein (TWP). The TWP meatless burger patty formulations bound using wheat gluten, dehydrated egg whites, and xanthan gum out were preferred to commercially available Textured Plant Protein (TVP) patty in the sensory analysis. The formulae take advantage of whey's mild flavor resulting in a product more desirable than the commercially available TVP meatless patty made primarily from soy protein concentrate.

3. Anticipated Problems/Delays:

None

Publications:

Development and Sensory Analysis of a Textured Whey Protein Meatless Burger. Submitted to J. Food Sc.

Theses:

Published Abstract:

Presentations:

Patent/Invention Disclosures:

Technology Transfer Activities
For information on licensing contact:

Visitors Hosted:

Western Dairy Center Report Addendum
December 31, 1997

Confidential - For Center Director's Use Only
(Release of this information requires prior approval by principal investigator)

Provide summary of significant advances in project that you wish to remain confidential at this time. For example, new methodology that you do not with to release until the current work is completed; information on invention disclosures or patents filed; activities conducted to transfer the technology to industry,or licensing agreements that are underway.

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

Invention Disclosures: (Title, Date)

Patents: (Title, Date, #)

Licensing Activities:

Discoveries:

Western Dairy Center

Project Report

Reporting Period January 1, 2000 — June 30, 2000

Principal Investigators: **Rajiv Dave**
Co-Investigators:

Project Title: **Effect of plasmin, plasmin inhibitor and plasmin activator on breakdown of b-casein and its correlation with melt and rheology of Mozzarella cheese.**

Institution's Project #: 00113

Project Completion Date: 12/31/00

National Research Plan (1997): Priority: milk/whey powder **Goal:** 2 **Tactic:** 2

Modifications to Project/Budget:
The three replications taken on making cheese with plasmin added milk failed and hence the project has been diverted and hence specific project objectives have been modified. However, the main objective of establishing correlation of melt with the breakdown of beta-casein has not been changed.

Project Objectives: (Include any revisions to objectives)

Because the plasmin was not binding properly and the plasmin inhibitor 6-amino hexanoic acid did not work as expected in the direct acid Mozzarella cheese system, a change in experiment plan was required. Thus, instead of testing plasmin and plasmin inhibitor, we will be now studying the effect of coagulant (1x and 6x) sure curd (from *Endothia parasitica*) v/s rennet and also study the effect of type of starter culture on rheology and melt of Mozzarella cheese. Sure curd is specific to beta-casein. The specific objectives are to study:

(a) the effect of rennet and sure-curd (from *Endothia parasitica*) at 1x and 6x concentration on proteolysis, rheology and melt of part-skim Mozzarella cheese

(b) the effect of proteolytic (*S. thermophilus* and L. *delbrueckii* ssp. *bulgaricus*) and non-proteolytic (S. *thermophilus*) starter culture on proteolysis, rheology and melt of part-skim Mozzarella cheese.

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

The project has just started and hence there are no findings to be released to the

public.

1. Significant Progress against Objectives:

For this project, student Ms. Pragati Sharma was awarded with the Graduate Research Assistantship and has started her work on this project. The final agreement for this project was made from March, 2000. Hence, all the major experiments/replications are underway. The three replications that were taken with plasmin and plasmin inhibitor did not show any specific trend and hence the project was modified to achieve the breakdown of beta-casein by using proteolytic culture and sure curd coagulant from *Endothia parasitica*. The preliminary trials have been completed and the rate of starter addition, maximum rate of coagulant that we can add, rheology parameters (fall in height and measurement of complex and elastic modulus) and melt (using computer imaging) etc. have been standardized for the final replications, which are already underway. From the initial trials, it was found that there is a difference in melt and rheology of cheese made with different coagulants at various concentrations. We have started our actual replications now and hoping to get the exact idea as to how the proteolysis is correlated to melt and rheology of cheese.

Also, Dr. Rajiv Dave has completed writing of 3 research papers and handed in 2 to Dr. McMahon for further modifications before final submission. One of the research paper on whey project has already been accepted by J. Dairy Sci. and will be published soon. This was as a part of his obligation with Utah State University, when he worked as a post-doctoral researcher in Dr. McMahon's laboratory.

2. Significant Conclusions:

None at this stage.

3. Anticipated Problems/Delays:

None at this stage.

Publications:

Will be submitted by the end of this year.

Theses:

None at this stage

Published Abstract:

None at this stage

Presentations:
None at this stage

Patent/Invention Disclosures: None

Technology Transfer Activities
For information on licensing contact:

Visitors Hosted: None

Western Dairy Center

Project Report

Reporting Period January 1, 2000 — June 30, 2000

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

Project Title: **Effects of microbial exopolysaccharide on functionality in high moisture cheese.**

Institution's Project #: **00115**

Project Completion Date: **12/31/00**

National Research Plan Priority: cheese **Goal:** 10

Modifications to Project/Budget:
none

Project Objectives: (Include any revisions to objectives)

1. To characterize the effect of a capsular exopolysaccharide on the firmness of increased moisture stirred curd cheese.
2. To determine whether functional properties of shredded cheese are maintained when cheese is produced with a capsular exopolysaccharide-producing starter culture.

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

How well a cheese can be shredded depends upon its firmness and adhesiveness. If cheese is too soft or sticky, it doesn't form uniform shreds and may foul shredding equipment. This project will determine whether a capsule (exopolysaccharide)-producing starter culture can be used to improve shreddability, as measured by firmness, in high moisture American style cheese. To accomplish this, we will manufacture stirred curd cheeses with equivalent moisture levels using MR-1C or a nonexopolysaccharide-producing starter. The shreddability of each cheese will then be determined and melt properties of the shredded cheese measured during storage.

1. Significant Progress against Objectives:

We have developed methodology to manufacture stirred curd cheeses with equivalent moisture levels using MR-1C or a nonexopolysaccharide-producing starter, and have begun to investigate firmness and shreddability of these cheeses.

2. Significant Conclusions:
see progress, above

3. Anticipated Problems/Delays:
none

Publications:
none

Theses:
none

Published Abstract:
none

Presentations:
none

Patent/Invention Disclosures:
none

Technology Transfer Activities
For information on licensing contact:
Jeff Broadbent or Donald McMahon

Visitors Hosted:
none

Western Dairy Center Report Addendum
June 30, 2000

Confidential - For Center Director's Use Only
(Release of this information requires prior approval by principal investigator)

Provide summary of significant advances in project that you wish to remain confidential at this time. For example, new methodology that you do not with to release until the current work is completed; information on invention disclosures or patents filed; activities conducted to transfer the technology to industry,or licensing agreements that are underway.

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:
see above

Western Dairy Center

Project Report

Reporting Period April 1, 2000 — June 30, 2000

Principal Investigators:	**Jeff Broadbent**
Co-Investigators:	Drs. James Steele and Bill Wendorff, University of Wisconsin-Madison
Project Title:	**Production of intensely flavored Cheddar-type cheeses by adjunct cultures.**
Institution's Project #:	**00116**
Project Completion Date:	**12/31/00**

National Research Plan Priority: cheese **Goal:** 3.3

Modifications to Project/Budget:
none

Project Objectives: (Include any revisions to objectives)

1. The construction of strains of *Lactobacillus casei* which produce elevated levels of diacetyl.
2. Construction of strains of *Lactobacillus casei* which over-express a bacterial lipase known to enhance cheese flavor.
3. Manufacture processed cheese from Cheddar cheese having significantly elevated levels of free fatty acids or furanones and pyrazines.

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

Use of cheese as an ingredient is in part dependent on the impact of the cheese on final product flavor. Process cheese is a significant cheese group and an excellent model system to study carry through of specific flavor compounds into natural cheese-derived products. This project seeks to utilize flavor adjunct lactic acid bacteria to produce elevated levels of specific flavor compounds in natural cheese and then determine the impact of those flavor compounds in processed cheese.

1. Significant Progress against Objectives:

Objective 1. Primers designed based upon pyruvate-formate lyase genes from other microorganisms have been utilized to generate an amplicon from *Lb. casei*. Preliminary analysis of the DNA sequence of this amplicon indicates that it is a pyruvate-formate lyase gene.

Ojective 2. Attempts to develop a plate assay for the identification of lipase positive clones of *Lb. casei* have been unsuccessful. Therefore, we have

changed to a flood plate screen in *Escherichia coli* to identify lipase/esterase genes from *Lb. casei* Lila. Initial results indicate that we have identified at least one, if not more, lipase/esterases from this organism.

2. Significant Conclusions:

see progress, above

3. Anticipated Problems/Delays:

none

Publications:

none

Theses:

none

Published Abstract:

none

Presentations:

none

Patent/Invention Disclosures:
none

Technology Transfer Activities
For information on licensing contact:
Jeff Broadbent

Visitors Hosted:
none

Western Dairy Center Report Addendum
June 30, 2000

Confidential - For Center Director's Use Only
(Release of this information requires prior approval by principal investigator)

Provide summary of significant advances in project that you wish to remain confidential at this time. For example, new methodology that you do not with to release until the current work is completed; information on invention disclosures or patents filed; activities conducted to transfer the technology to industry,or licensing agreements that are underway.

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:
see above