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Western Dairy Center

Western Dairy Center Annual Report

1998

WESTERN DAIRY CENTER

WESTERN DAIRY CENTER

ANNUAL REPORT

1998

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Western Dairy Center

Project Report

Reporting Period June 1, 1997 — December 31, 1998

Principal Investigators: Dr. Jeffery Broadbent, Utah State University

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

Dr. Craig Oberg, Weber State University

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

Institution's Project #: 97079

Project Completion Date: 12-31-99

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

Modifications to Project/Budget:

None

Project Objectives: (Include any revisions to objectives)

Understand the influence of microbial exopolysaccharides on Mozzarella
cheese moisture status and the relationship of moisture to cheese
composition and functionality.

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

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

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

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

Mozzarella cheese functionality is significantly affected by cheese
moisture level, and increasing moisture content has been used to improve
the melting properties of low fat Mozzarella cheese. Previous work by our
group has shown that exopolysaccharide (EPS)-producing starter cultures can

be used to significantly increase the moisture content of lower fat Mozzarella cheese. We have identified a culture, *Streptococcus thermophilus* MR-1C, that produces a large capsular EPS, and have shown that this capsule is involved in cheese moisture retention. Ongoing experiments are characterizing the structure of the MR-1C EPS and the genes that encode its biosynthesis. This project will provide basic information needed to understand the influence of EPS on cheese quality and functionality

1. Significant Progress against Objectives:

Research to date continues to address objectives 1 and 2. Our initial analysis of the composition of the MR-1C EPS by GC-mass spectroscopy indicated the polymer had a novel octomeric repeating unit composed of galactose, rhamnose and fucose in a 5:2:1 ratio. A more recent chemical analysis, performed in collaboration with polymer chemists at Nestlé, Inc., have confirmed the presence of these 3 sugars but suggest that the actual ratios may differ. This discrepancy is not a concern, however, because ongoing nuclear magnetic resonance experiments at Nestlé will soon provide us with a definitive view of the MR-1C EPS structure.

Efforts to isolate and sequence the MR-1C EPS gene cluster have also been relatively successful. We have completed nucleotide sequence analysis of approximately 9-kb of the MR-1C *eps* gene cluster. This region includes genes involved in the regulation of EPS biosynthesis (*epsA*), EPS polymerization and export (*epsC*, *epsD*) and several glucosyltransferases that are likely involved in assembly of the basic repeating unit (*epsE*, *epsI*, *epsF2*, and *epsQ*). We have also cloned and begun to sequence a large (> 14-kb) fragment of the MR-1C chromosome that includes *epsF* and downstream sequences. Nucleotide sequence analysis will now be used to determine whether this clone includes the remainder of the MR-1C *eps* gene cluster. As that sequence is determined, we will utilize it to identify a strategy to assemble the entire gene cluster into a lactic acid bacteria cloning vector. The complete *eps* gene cluster will then be transformed into EPS- Mozzarella starter cultures and the influence of these constructs on the moisture level and functional properties of low-fat Mozzarella cheese will be evaluated.

2. Significant Conclusions:

Our data demonstrate that the *S. thermophilus* MR-1C EPS is a novel polymer with unique commercial applications. Our research has also provided new insight into the physiology of galactose catabolism by *S. thermophilus* and that result is under further investigation.

3. Anticipated Problems/Delays:

none

Publications:

- 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:

- 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., D.J. McMahon, and C.J. Oberg. 1998. Practical Considerations in the Use of Exopolysaccharide-producing Cultures. Invited oral presentation for the Marshall/Rhodia, Inc. Italian and Specialty Cheese Conference, Sept. 16-17, Madison, WI.
- Broadbent*, J.R. 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. EPS production in *Streptococcus thermophilus*: physiology, biochemistry, and genetics. 1997. Invited oral presentation for the Marshall/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 a culture manufacturer to evaluate the cultures for commercial production. For information on licensing contact:
Jeff Broadbent or Carl Brothersen

Western Dairy Center

Project Report

Reporting Period July 1, 1997 — December 31, 1998

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: 12-31-99

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:

The ability to address population dynamics between starter, non-starter, and adjunct bacteria during cheese ripening requires methodology that will allow us to monitor changes in that population, over time, at the strain level. To accomplish this, we have developed methodology for random amplified polymorphic DNA (RAPD) fingerprinting by the polymerase chain reaction (PCR). Our results confirm that we can differentiate between individual strains of *Lactococcus lactis*, *Lactobacillus casei*, and *Lactobacillus helveticus*. We have also been able to isolate bacterial DNA from commercial cheese and use this DNA as a template for the amplification of 16S rRNA genes that will allow us to speciate bacteria that may dominate NSLAB population but cannot be cultured in the laboratory. With these methodologies in place, 50% reduced-fat cheese was manufactured at UW-Madison in early November, and we are now heavily involved in the analysis of NSLAB population dynamics as outlined under objectives 1, 2, and 4.

2. Significant Conclusions:

RAPD-PCR is an effective method for the analysis of NSLAB population dynamics in ripening cheese.

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

Project Report

Reporting Period July 1, 1997 — December 31, 1998

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: 12-31-99

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)

The overall objective of this proposal is to identify and characterize enzymes produced by starters and flavor adjunct bacteria which are responsible for the production or degradation of bitter peptides in Cheddar cheese.

- Objective 1 Define the contribution of starter CEP specificity on peptide pools and bitterness in Cheddar cheese.
- Objective 2 Develop a cheese-based test for bitterness in Cheddar cheese and establish factors that influence sensory perception of bitterness in Cheddar cheese.
- Objective 3 Determine bitter taste thresholds for β -CN (f193-209) and α_{s1} -CN (f1-9).
- Objective 4 Define the contribution of *Lactobacillus helveticus* CNRZ32 peptidases to degradation of β -CN (f193-209) and α_{s1} -CN (f1-9).
- Objective 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 be 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:

Under objective 1, Dr. Broadbent's lab successfully cloned the bitter *L. lactis* S3 proteinase and has constructed a series of isogenic *L. lactis* strains that differ only by the expression of a group a, e, or h (bitter) cell envelope proteinase. The proteinase specificity of these constructs was confirmed by *in vitro* incubation with α_{s1} -CN (f1-23) 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 we are now using these cheeses to establish the effect of starter proteinase specificity in peptide accumulation and bitterness.

Under Objective 2, Dr. Brennan assembled a trained bitter sensory panel and determined taste thresholds for the α_{s1} -CN (f1-9) and β -CN (f193-209) bitter peptides in water as outlined under objective 3. Experiments are now underway to establish the taste thresholds of these peptides in our model cheese system.

Research under objective 4 by Dr. Steele's group has clearly shown that the *Lactobacillus helveticus* CNRZ32 general aminopeptidase and other enzymes can degrade the bitter peptide β -CN (f 193-209) under cheese pH and salt levels, and HPLC studies are underway to characterize the specificity of these enzymes on this peptide and α_{s1} -CN (f 1-9). Once those experiments are complete, Dr. Steele will construct the SK11 peptidase overexpression system outlined under Objective 5.

2. Significant Conclusions:

Dr. Brennand's trained sensory panel has confirmed that the α_{s1} -CN (f1--9) and b-CN (f193-209) peptides are bitter at concentrations well below those found in cheese. This finding supports our hypothesis that starter CEP's like the group h enzyme from *L. lactis* S3 which preferentially liberate α_{s1} -CN (f1--9) contribute to bitterness in Cheddar cheese. 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:

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. 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 — December 31, 1998

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: 12-31-99

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)

The overall objective of this proposal is to identify and characterize key enzymes and mechanisms which are primarily responsible for the conversion of aromatic amino acids into off flavor compounds in low-fat Cheddar cheese.

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

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

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 has been completed. Metabolic cross-feeding studies for Trp, Phe, and Tyr confirmed that these reactions are active under simulated cheese conditions (pH 5.2, 4% NaCl, no carbohydrate, 13-15°C). Experiments to address objective 2 are now well underway. Thus far we have been able to isolate the *L. casei* gene encoding the NAD(H)-dependent D-2-hydroxyisocaproate dehydrogenase (D-HicDH), and work is underway to clone the gene encoding the aromatic aminotransferase (AATase). We are also working on the development of an efficient electrotransformation system for *L. casei*, and have procured a temperature-sensitive integration vector for that bacterium, pV6004. The latter experiments are essential to our effort to create isogenic mutants which lack D-HicDH or AATase, and thereby establish the contribution of each enzyme to aromatic amino acid catabolism and off-flavor production in cheese.

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 off flavor compounds.

3. Anticipated Problems/Delays:

Our efforts to isolate the *L. casei* aromatic aminotransferase has been delayed by several unforeseen problems, particularly the finding that the bacterium we were originally using for this purpose was not a true strain of *L. casei*. We believe our problems have been overcome and hope that we will soon be successful in our effort to isolate this gene.

Publications:

Gummalla, S., and J.R. Broadbent. Tryptophan catabolism by *Lactobacillus casei* and *Lactobacillus helveticus* cheese flavor adjuncts. submitted.

Theses:

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

Published Abstract:

none

Presentations:

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, 1998

Principal Investigators: Bart C. Weimer

Co-Investigators:

Project Title: Investigate the role of metabolic cross-feeding between starter, *brevibacterium* and nonstarter cheese bacteria in the production or removal of aromatic off flavor compounds.

Institution's Project #: 97087

Project Completion Date: 12/31/98

National Cheese 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)

To investigate the role of metabolic cross-feeding between starter, *brevibacterium* and nonstarter cheese bacteria in the production or removal of aromatic off flavor compounds.

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

Brevibacteria don't produce aromatic off flavors from aromatic amino acids. Lactic acid bacteria produce many different compounds. Cross feeding of intermediates between dairy-related bacteria was demonstrated for *lactobacilli* and *brevibacteria*. While *lactobacilli* produce undesirable aromatic off flavors from aromatic amino acids *brevibacteria* may remove those compounds to compounds that are flavorless or energy during cheese ripening. In some cases this seems to be true, while in other cases, other bacteria, this is not the case. These observations are being verified with cheese from another project that is adding different adjunct bacteria.

B. linens BL2 degrades all the aromatic amino acids. This strain degrades Trp to kynurenine, anthranilic acid, and three unknown aromatic compounds. Kynurenine and anthranilic acid are not associated with known off flavor compounds. Phe is degraded to phenylacetic acid, and Tyr is degraded to non-aromatic compounds. *Lactobacilli* utilize

phenylacetic acid during metabolism. BL2 didn't utilize aromatic off flavors when added to cultures. When added to cheese BL2 significantly improved cheese flavor and did not increase the aromatic off flavors in trained and consumer taste panels. These observations indicate that BL2 is an acceptable adjunct flavor organism for Cheddar cheese.

1. Significant Progress against Objectives:

All objectives are being finished as proposed.

2. Significant Conclusions:

The total microflora of the cheese is important for the final flavor profile due to cross feeding. Further work to define the microflora beyond NSLAB is needed to determine the full impact of this finding.

3. Anticipated Problems/Delays:

none

Publications:

Ummadi, M. and B. C. Weimer. Tryptophan metabolism in *Brevibacterium linens* BL2 (submitted).

Ummadi, M. and B. C. Weimer. Use of *Brevibacterium linens* BL2 to reduce off flavor production in Cheddar cheese(submitted).

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

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.

Theses:

Madhvi Ummadi

Published Abstract:

none

Presentations:

Ummadi, M. and B. C. Weimer. 1998. Aromatic amino acid metabolism and cheese flavor. Utah Academy of Science.

Patent/Invention Disclosures:

Technology Transfer Activities

For information on licensing contact:

none

Visitors Hosted:

Western Dairy Center

Project Report

Reporting Period January 1, 1997 — December 31, 1998

Principal Investigators: Dr. Jeffery R. 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 Cheese 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:

Research to date has focused on objective 1. We have completed our nucleotide sequence analysis of the *L. helveticus* LH212 *groESL* operon and have confirmed that the *groESL* promoter is tightly regulated, at the transcriptional level, by heat shock. Northern analysis confirmed that constitutive expression of *groESL* is very low but transcription of the operon is induced more than 400% upon temperature upshift from 37 to 52C. As part of our effort to characterize the utility of this promoter, we have designed PCR primers that will allow us to insert the promoter upstream of the *E. coli gusA* gene in the lactic expression vector pNZ272. This will allow us to accurately quantify promoter activity in *L. helveticus* and *Lactococcus lactis*. In addition, we also discovered that the *L. helveticus groES* gene, like its counterparts in *L. zeae* and *B. subtilis*, utilizes the uncommon start codon UUG. The importance of UUG and other rare initiation codons in the genus *Lactobacillus* is unknown, but in *Escherichia coli* and (to a lesser extent) *B. subtilis*, non-AUG initiation codons act to limit the expression of a gene product at the translational level. For this reason, we have designed an additional set of PCR primers that will allow us to investigate the influence of a UUG versus AUG initiation codon on *gusA* expression in lactic acid bacteria. Once those experiments are complete, we will begin to work on objectives 2 and 3.

2. Significant Conclusions:

Results to date support our hypothesis that the *L. helveticus* LH212 *groESL* promoter may be a useful metabolic control switch for the development of a process-regulated gene expression system that could be used to closely regulate enzyme production in cheese starter bacteria

3. Anticipated Problems/Delays:

none

Publications:

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.

Broadbent, J.R., C.J. Oberg, and L. Wei. 1998. Characterization of the *Lactobacillus helveticus* groESL operon. *Res. Microbiol.* 149:247-253.

Theses:

none

Published Abstract:

Broadbent, J.R., L. Wei, D.J. McMahon, and C.J. Oberg. 1998. Characterization of the *Lactobacillus helveticus* groESL operon. *J. Dairy Sci.* 81(Suppl. 1):5.

Presentations:

Broadbent, J.R., L. Wei, D.J. McMahon, and C.J. Oberg*. Characterization of the *Lactobacillus helveticus* groESL operon. Poster presentation at the Ann. Mtg. Amer. Dairy Sci. Assoc. July 27-30, Denver, CO.

Patent/Invention Disclosures:

No patents/invention disclosures filed

Technology Transfer Activities Technology is available for licensing For information on licensing contact: Jeff Broadbent
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Visitors Hosted:

none

Western Dairy Center

Project Report

Reporting Period January 1, 1997 - December 31, 1998

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: 12/31/99

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

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

Modifications to Project/Budget:

None

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 a knowledge of the host components required for attachment and

phage entry. The outcomes of this proposal will enable the construction of new

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

1. Significant Progress against Objectives:

We have cloned by complementation, lactococcal genes required for infection of phage sk1, which is a phage of the species 936. We have partially sequenced the cloned genes, and have identified all of them by homologies to known genes in the data bases. There are four genes on one complementing piece of chromosome, and one gene on a second complementing piece of chromosome, which does not overlap the first piece. We are currently conducting experiments to determine which of the four genes on the first piece is responsible for the complementing phenotype.

We have also constructed insertion mutants of *Lactococcus lactis* that are resistant to a p335 species phage. We have screened 6 of these mutants and

found that they are defective in absorbing phage. We are currently screening more of the phage-resistant mutants to determine if any absorb phage normally. We plan to clone at least one of the genes responsible for the phage-resistant phenotype. However, progress on this goal has been on hold since last summer when the PI was able to do the bench work. We have been unable to secure a graduate student as planned for this part of the project.

Phage-resistant mutants of *L. lactis* C2 were isolated after challenge of *L. lactis* C2 with phage sk1. Two mutant strains, RMSK1/1 and RMSK1/3 that had cell wall compositions indistinguishable from that of *L. lactis* C2, and adsorbed phage sk1 particles, were chosen for further study. To identify the locus (or loci) of *L. lactis* responsible for this resistance to phage sk1, we complemented *L. lactis* RMSK1/1 and RMSK1/3 with a genomic library of *L. lactis* LM2301 DNA (LM2301 is a plasmidless derivative of C2). Two distinct genomic regions of *L. lactis* LM2301 DNA were identified that restored phage sensitivity to *L. lactis* strains RMSK1/1 and RMSK1/3 through genetic complementation. Complementing clones were sequenced. Sequence analysis of the first region revealed a lysozyme gene, several tRNA genes, and open reading frames with similarities to the Sun protein of *Bacillus subtilis*, phosphoprotein phosphatases, and protein kinases. Subclones of the complementing DNA were analyzed for their abilities to restore phage sensitivity to RMSK1/1. Sequence analysis of the second region contained an alcohol dehydrogenase gene. The complementation by this region was weaker and more variable so was not studied further.

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

DNA similar to that of temperate lactococcal bacteriophage of the p335 species was detected 307 bp distal to lysL. Phage with similar DNA include BK5-T, rlt, LC3, and Tuc2009. This region of similarity contains terminator sequences and regions involved in homologous recombination. In these phage, this region is located from 173 to 197 bp distal to the respective lysin genes. An alignment of these similar nucleotide sequences revealed a 266 base-pair stretch of DNA disrupting the region of similarity. The 266 base-pair intervening sequence contains two sets of inverted repeats. The symmetry of the inverted repeats in the 266 bp suggests that a transposition event occurred in this region.

Several lines of evidence indicate that this region does not contain an intact integrated prophage, despite the fact that lysogeny is the rule rather than the exception for lactococcal strains. Indeed, strain C2, the parent of strains RMSK1/1 and LM 2301, harbors a prophage that produces a small, isometric virion upon induction with UV light. Although DNA similar to that of temperate lactococcal bacteriophages of the p335 species was detected distal to lysL, the lysin enzymes of these temperate phages are less similar to LysL than the proteins discussed above. In addition, the regions bordering lysL were not similar to the att regions of temperate phages, and the regulatory sequences

present in lytic and temperate phages of *Lactococcus lactis* were not present in the 5832 bp sequenced. Also, the region of similarity to temperate phage of the p335 species appeared to be interrupted by a transposition event. The homologies of *lysL* with *lys* genes from other phages, and ! the similarities with regions of temperate phage of the p335 species suggests that this may be the site on an ancient prophage integration

Construction of modified versions of the phage *sk1*-sensitivity region.

Complementing DNA was subcloned, using phage sensitivity to assay for complementation. The region responsible for the complementation was narrowed to *LysL* and about 1300 bp downstream of this locus. The region further downstream did not complement. We constructed a modified version of the *lysL* gene that contains nonsense codons in all three frames and a unique restriction site as a distinguishing marker for the modified version of *lysL*. This nonsense allele complemented the phage resistant mutant. Our preliminary interpretation is that *LysL* is not responsible for the complementation, but the region downstream of this locus is. We are currently generating site-directed deletions in other regions of the subcloned DNA to test our preliminary interpretation. We have made the deletion constructs and placed them in the integration vector for allelic exchange, but have not yet exchanged the wild type and deletion alleles.

2. Significant Conclusions:

A phage *sk1*-resistant phenotype that raises the minimum calcium concentration required for phage infection to 20mM may not be complementable. The extra complexity of the calcium concentration-dependency appears to impose a risk that this phenotype may not lead to host factors that are directly related to phage infection.

As a consequence of this, different phenotypes are now being complemented.

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

3. Anticipated Problems/Delays:

We have been unable to secure a graduate student to work on this project as proposed in the project plan. There may be an opportunity to assign one of my lab technicians to the project, which would expedite the work.

Publications:

Garbutt, K.C., J. Kraus, and B.L. Geller. 1997. Bacteriophage-Resistance in *Lactococcus lactis* Engineered by Replacement of a Gene for a Bacteriophage

Receptor. J. Dairy Sci. 80:1512-1519.

Geller, B.L., J. Kraus, M.D. Schell, M.J. Hornsby, J.J. Neal, and F. Ruch. 1998. High-titer, Phage-Neutralizing Antibodies in Bovine Colostrum prevent Lytic Infection of Lactococcus lactis in Phage-Contaminated Milk Fermentations. J. Dairy Sci. 81:895-900.

Kraus, J. and B.L. Geller. 1998. Membrane Receptor for Prolate Phages is Not Required for Infection of Lactococcus lactis by Small or Large Isometric Phages. J. Dairy Sci. 81:2329-2335.

Theses:

None

Published Abstract:

None

Presentations:

None

Patent/Invention Disclosures:

We have filed an Invention disclosure with the Technology Transfer Office at Oregon State University. We have signed confidentiality agreements with 3 major culture suppliers, with the objective of negotiating an agreement with one or more of them for licensing this invention.

Technology Transfer Activities

For information on licensing contact:

Visitors Hosted:

None

Western Dairy Center

Project Report

Reporting Period January 1, 1998 — December 31, 1998

Principal Investigators: Donald J. McMahon, Utah State University

Co-Investigators: Jeffery Broadbent
Craig Oberg

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

Institution's Project #: 98092

Project Completion Date: December 31, 1999

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

Understand role of moisture and proteolysis on physical and functional properties of cheese.

Modifications to Project/Budget:

None

Project Objectives: (Include any revisions to objectives)

Overall Objective: Understand the influence of microbial proteolytic systems which have variable effects on the rheological properties of Mozzarella cheese.

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

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

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

The purpose of this project is to control the functionality of Mozzarella cheese by understanding the role of starter culture proteolysis in the development of melting properties. This project will investigate (1) the diversity that exists in the proteolytic systems of important thermophilic starter cultures, and (2) how these differences may influence Mozzarella cheese functionality. Strains of lactobacilli and *Streptococcus thermophilus* will be screened for proteinase activity, specificity toward α_{s1} -casein (f 1-23) and their ability to degrade individual intact caseins. Strains found to represent distinct proteinase enzymes within lactobacilli species will then be added to cheese slurries and

Mozzarella cheese to investigate the effect of these enzymes on proteolysis and cheese functionality. Knowing characteristics of starter culture proteolytic systems would provide a sound basis for starter culture selection; so that functionality can be customized to meet customer requirements. This information would then make it possible to develop starter culture systems for optimum manufacture of both full and lower fat Mozzarella cheese.

1. Significant Progress against Objectives:

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

Objective 1, Step 2 - Eighteen strains of lactobacilli (*Lactobacillus helveticus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) have been characterized for proteolysis using the α_{s1} -casein (f 1-23) method. Analysis for the remaining characterized strains is underway.

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

2. Significant Conclusions:

Preliminary data for the α_{s1} -casein (f 1-23) method indicates two possible types of proteolysis can be found in lactobacilli and differences in a preferential cleavage pattern of the α_{s1} -casein (f 1-23) for various strains into the final peptide products can be used for strain characterization.

3. Anticipated Problems/Delays:

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

Publications:

None at present.

Theses:

None

Published Abstract:

None at present.

Presentations:

None at present.

Patent/Invention Disclosures:

Technology Transfer Activities

For information on licensing contact:

Visitors Hosted:

None

Western Dairy Center

Project Report

Reporting Period January 1, 1997 — December 31, 1998

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

Salaries/Benefits line items for postdoctoral fellow was modified to include housing and travel expenses. No change to total personnel costs incurred.

Project Objectives: (Include any revisions to objectives)

1. Determine the contribution of alpha₁-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 alpha₁-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 alpha₁-casein or beta-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

durrring 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:

Three 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, were commenced in October. 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 heating 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. Statistical analyses of the collected data is expected to be completed in the first half of 1999.

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. It appeared that little hydrolysis of beta-casein occurred until all intact alpha-S1 casein was hydrolyzed. 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.

3. Anticipated Problems/Delays:

We do not anticipate any problems or delays in the project now and expect to complete the second objective of this study by December 1999.

Publications:

Nil

Theses:

Nil

Published Abstract:

Nil

Presentations:

Nil

Patent/Invention Disclosures:

Technology Transfer Activities

For information on licensing contact:

Not applicable at this time.

Visitors Hosted:

Alan Foegeding, North Carolina State University, visited Utah State University in December 1998 and presented a seminar on rheology.

Western Dairy Center

Project Report

Reporting Period January 1, 1998 — December 31, 1998

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: December 31, 1999

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.

1. Significant Progress against Objectives:

The objectives outlined were accomplished. Through systematic

experimentation we were able to narrow the processing conditions for UHT milk by electroheating. The sensory evaluation, flavor analysis, and the protein denaturation experiments were highly desirable for the electroheated milk compared to the commercial milk. The flavor attributes of electroheated milk were in general favorable for Electroheated milk compared to the commercial variety. Additional experiments are planned for further comparison with other samples and to determine the optimum processing conditions.

2. Significant Conclusions:

Sensory analysis strongly indicates that the flavor of UHT milk by electroheating is highly desirable to one commercial variety.

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, 1997 — December 31, 1998

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: 6-30-98

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 whiteness, texture and flavor.

Modifications to Project/Budget:

None

Project Objectives: (Include any revisions to objectives)

To develop a suitably active immobilized protease for use in skim milk whitening. To characterize the immobilized enzyme catalyst for operational stability and productivity in a fluidized-bed reactor with respect to skim milk whitening.

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

Covalently immobilized milk clotting enzymes have been used to whiten skim milk. The increase in whiteness value obtained with immobilized enzymes was significantly higher than the whiteness value obtained with soluble enzymes. We believe the use of immobilized enzymes offers more control over the whitening process, which allows us to reach a higher whiteness level without coagulating the skim milk.

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, nonporous ceramic and glass beads, and controlled-pore glass (CPG) beads, to compare the immobilization efficiency. Acid-cleaned beads were silanized with 3-aminopropyltriethoxysilane at pH 4.0 and 70 °C. Aminoethyl beads were activated with 2% (v/v) glutaraldehyde in 50 mM sodium phosphate buffer, pH 6.5.

Enzymes, porcine pepsin (Sigma) and chymosin (Chynax Ultra, Chris Hansen), were coupled to beads either directly or via a crosslinker protein, bovine serum albumin (BSA). 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) to remove all traces of free enzyme.

For immobilization involving a crosslinker, a solution of 750 mg BSA in 25 ml 50 mM sodium phosphate buffer, pH 6.5 was mixed with the beads for 1 hr at room temperature. Excess protein was removed with distilled water and the BSA-coated beads were reduced by the addition of 100 mg of NaBH₄. The reduced, protein-coated beads were activated by treatment with 2% glutaraldehyde as already described. After completely washing off excess glutaraldehyde, enzyme solution was added and reduced with NaBH₄. The enzyme-coupled beads were thoroughly washed with SMUF buffer to remove free enzyme.

Whitening skim milk with immobilized proteases

The immobilized enzyme preparations were housed in 25 x 10 cm glass columns. About 2.5 ml pasteurized skim milk was circulated through the reactor via a peristaltic pump at room temperature. Sodium azide, 0.02% was added to the milk to prevent bacterial growth. Before starting the experiment, the biocatalyst was washed with 25 ml skim milk which was then discarded. During experiments, 20 ml of skim milk were collected at appropriate intervals for color determination, L value, using a Hunterlab D25D22A color meter.

Fluorometric measurement of immobilized enzyme activity

Soluble and immobilized enzyme activity was determined fluorometrically using Renin Substrate 1 (Molecular Probes) in 0.05 M sodium phosphate buffer (pH 6.0). The enzyme activity was expressed as relative fluorescence unit (RFU) which were measured by monitoring the increase in fluorescence signal at 490 nm for 7-8 min.

Determination of soluble activity of immobilized enzymes

To monitor the leaching of enzyme from the matrix, the enzyme treated milk samples were collected and acidified to coagulate the casein. Aliquots of the whey were adjusted to pH 6.0 with 1 N NaOH then assayed for enzyme fluorometrically.

2. Significant Conclusions:

Table 1 shows the influence of support type on the activity of the immobilized protease. Enzymes were immobilized without the use of a crosslinker. CPG showed a significantly higher immobilized enzyme activity compared to nonporous ceramic and glass beads. CPG beads also showed a significant amount, in some cases greater than a 10 fold increase, in nonspecifically adsorbed protein.

To reduce the amount of nonspecifically adsorbed enzyme, we retain a high enzyme activity, chymosin and pepsin were immobilized onto CPG using a crosslinker (BSA) and a reducing agent (method 2). Increases in immobilized enzyme stability have been observed after treatment with a reducing agent which may result from a reduction of the more labile imino bonds between glutaraldehyde and enzyme. Table 2 compares the activity of immobilized enzymes as influenced by immobilization method. The total activity of immobilized enzymes was the same with both methods, but the reducing step decreased the amount of nonspecifically adsorbed enzyme (shown as soluble enzyme) by about 10 fold.

Immobilized chymosin and pepsin, method 2, were able to whiten skim milk to an L value of 1% fat milk (Table 2). The time to reach the maximum L value was significantly longer with immobilized pepsin. Table 3 also shows that soluble pepsin is more effective at whitening skim milk, but the time for the reaction is significantly longer compared to chymosin. Soluble enzyme type and enzyme concentration have significant effects on the level of skim milk whiteness and the time it takes to reach maximum whiteness.

The increase in L value with immobilized enzymes was significantly higher than the L value obtained with the soluble enzyme (Table 4). We believe the use of immobilized enzymes offers more control over the whitening process, which allows us to reach a higher whiteness level without coagulating the skim milk.

3. Anticipated Problems/Delays:

Publications:

None

Theses:

None

Published Abstract:

Immobilized pepsin for skim milk whitening. X. Li and M.K. Valsh. 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 January 1, 1997 — December 31, 1998

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: 12/31/99

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 nutrients).

Modifications to Project/Budget:
None

Project Objectives: (Include any revisions to objectives)

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

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

Prosaposin plays a broad role in the development, maintenance, and repair of the nervous system. This protein has been shown to form stable complexes with gangliosides, therefore, our objective was to develop a bioselective adsorption matrix for the affinity purification of prosaposin from whey. Gangliosides were organically extracted from bovine buttermilk and coupled covalently to controlled-pore glass beads. WPC and WPI were used as the source of prosaposin. After loading the matrix with whey protein sample, the matrix was washed with various buffers and two 66 kDa glycoproteins were

eluted with sodium phosphate pH 7. These proteins were further purified by preparative electrophoresis and are being identified by N-terminal protein sequencing.

1. Significant Progress against Objectives:

Materials and Methods:

Ganglioside Extraction

Gangliosides were extracted from fresh bovine butter milk (Broaden Butter, Logan, UT). Butter milk was diluted 1 to 10 with water and ultrafiltered using prep/scale-TFF 2.5 ft² cartridge membrane (10 KDa) (Millipore) to remove lactose. The efficiency of lactose removal was confirmed with a lactose enzymatic bioanalysis kit (Boehringer Mannheim). Moisture, protein, minerals, and lipid content of lactose-free buttermilk was determined by oven, Kjeldahl, ash, and Majonnier. Lactose-free buttermilk was freeze dried and gangliosides were extracted using 20 vol. of organic mixture of chloroform: methanol: water (40: 80: 30 vol.) per gram dry solids. The sample was centrifuged at 11,000g 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 (Jourdian et al., 1971).

Covalent Immobilization

Controlled-pore glass (CPG) beads (2000A, 120-200 mesh) (Sigma) were derivatized with 3-aminopropyltriethoxy silane and succinylated with succinic anhydride according to Walsh and Swaisgood (1996). Acetic anhydride (10 %, by vol.) in acetone was used to cap excess amino groups. Gangliosides were saponified to produce the lyso-derivative containing free amine group by the reflux boiling method (Tayot et al., 1981). The OPA method (O-phthalaldehyde) as described by Weimer and Oberg (1989) was used to confirm saponification of gangliosides. The carboxyl matrix was reacted with sulfo-NHS (Pierce Chemical Co.) in 0.01 M MES (pH 6.0) containing water-soluble carbodiimide (EDC). The lyso-gangliosides, dissolved in MES buffer (pH 6) were circulated over the sulfo-NHS matrix to couple via amide bond for 122 hours at 4 C using a peristaltic pump. Hydroxylamine (10 mM) was added to quench the reaction by hydrolyzing any unreacted NHS present. This reagent also served to cap the unreacted carboxyl groups to prevent nonspecific whey protein binding. The extent of ganglioside immobilized was determined by measuring the amount of sialic acid on the matrix (Jourdian et al., 1971).

Prosaiposin Purification

WPC (Cache Valley Dairy, Logan, UT) or WPI (Avenmore), 1% (wt/vol) in 50 mM sodium acetate were used as the starting material.

Immobilized gangliosides were packed into a stop-flow column and equilibrated with sodium acetate buffer (pH 4). Samples were applied to the column and circulated for 5 minutes at room temperature. Prosaposin was purified by batch mode using four different buffers; A) sodium acetate pH 4 containing 1 M NaCl; B) sodium acetate, pH 4; C) sodium phosphate pH 7; D) sodium phosphate pH 7 containing 1 M NaCl. After incubation for 5 minutes in each buffer, the eluting proteins were collected and each fraction was analyzed by non-reducing SDS-PAGE and densitometer scanning (Alpha Image). Eluant D was also analyzed by preparative electrophoresis. Glycoproteins present in eluant D were purified with immobilized Concanavalin A (Sigma).

2. Significant Conclusions:

Ganglioside Extraction

Fresh buttermilk contained 87.9% moisture, 8.6% crude protein, 2.7% lipid, and 0.365% minerals. Lactose was removed from buttermilk to prevent browning during saponification. The lactose content was reduced 100 fold (6.6 mg/L final concentration) and the ganglioside:protein ratio was 400 (0.019 initial ratio) in ultrafiltered, organic extraction samples.

Prosaposin Purification

Figure 1 shows the nonreducing SDS-PAGE analysis of proteins purified from whey. Sodium acetate containing salt was efficient in removing lactoferrin from the sample (Figure 1, Lanes 2, 4 and 6). Lactoferrin was eluted from the column with all buffers containing salt. Two proteins, pp1 and pp2, with slower mobility than bovine serum albumin (BSA) appear in eluants C and D (Figure 1, Lanes 7 and 8) which are not visible in the starting sample (Figure 1, Lane 1). These proteins have the expected location of prosaposin.

Preparative electrophoresis was used to purify pp1 and pp2 from eluant C. Figure 2 shows an SDS-PAGE analysis of proteins purified by preparative electrophoresis. Under reducing conditions, pp1 and pp2 have approximately the same molecular weight, 66 kDa, which is also the molecular weight as BSA. Yet, under nonreducing conditions, the proteins differ in mobilities. Since prosaposin is a glycosylated protein, Concanavalin A was used to purify the proteins eluting with buffer D. Figure 2, Lane 4 shows the glycoproteins present in this sample. Both pp1 and pp2 are glycosylated.

Western analysis using human anti-saposin C antibody was shown to bind BSA, pp1 and pp2, and lactoferrin, therefore pp1 and pp2 are being analyzed by N-terminal protein sequencing for identification.

References:

Jourd'hean, G. W., L. Dean, and S. Roseman. 1971. The sialic acids. *J. Biol. Chem.* 246 (2): 430-435.

Tayeb, J.L., J. Holmgren, L. Svennerholm, M. Lindblad, and M. Tardy. 1981. Receptor- specific large-scale purification of cholera toxin on silica beads derivatized with lyso-GM1 ganglioside. Eur. J. Biochem. 113: 249-258.

Walsh, M. K. and H. E. Swaisgood. 1996. Investigating the use of the chymosin- sensitive sequence of kappa- casein as a cleavable linker site in fusion proteins. J. Biotechnol. 45: 235-241.

Weimer, B. C. and C. J., Oberg. 1989. Comparison of classical ion exchange amino acid analysis and O-phthaldialdehyde methods to characterize proteolysis by lactobacillus bulgaricus J. Dairy Sci. 72: 2873-2876.

3. Anticipated Problems/Delays:

None

Publications:

Affinity purification of prosaposin from whey. S.H. Nam and M.K. Walsh.
In preparation for JDS.

Theses:

Published Abstract:

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

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. Development of a Bioselective Matrix for the Purification
of Prosaposin from whey. UASAL April 3, 1998.

Patent/Invention Disclosures:

Technology Transfer Activities
For information on licensing contact:

Visitors Hosted:

Western Dairy Center

Project Report

Reporting Period January 1, 1997 — December 31, 1998

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)

Dairy products are generally well-accepted food additives and there is a potentially large market for a textured whey protein (TWP) as an extender of ground meat products. We have investigated the influence of extruder configurations, temperature, pH, and added calcium on the extent of crosslinking in TWP samples. The extruder was configured with an expanded mixing section at 25°C to allow adequate mixing of dry sample and water. A long cooling die was constructed to produce a thin ribbon of extrudate. The extent of protein crosslinking in products containing 70% WPC and 30% carbohydrate were determined and compared to textured vegetable protein (TVP). Addition of acid, base and calcium chloride dihydrate during extrusion altered the pH of the extrudates, and affected product solubility. The results suggest that by altering extruder and raw material conditions it may be feasible to produce texturized WPC suitable for use in coarse-ground meat products.

1. Significant Progress against Objectives:

Materials

For each extrusion run, dry mixtures of 2/3 WPC 80 (80% protein) (American Meat Packers Cooperation, Ames IA) and 1/3 cornstarch (purchased locally) were used. WPC 80 contains 80% protein, 4.6% fat, 4.5% ash (0.294% calcium), 4.4% CHO and 4.2% moisture. Calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$), NaOH, and HCl were purchased from Malinkrodt, Paris, KY. In all experiments, Textured Procon, a textured soy protein concentrate (Central Soya, Fort Wayne, IN) was used as a control.

Extruder

Three treatments were investigated in multiple extrusion runs to determine the effects of temperature, pH and calcium on the solubility of the TWP. Extrusion was conducted on an APV Baker MPF19 twin-screw extruder. The extrusion parameters; screw speed of 200 rpms, feed rate of 23 g/min, water-flow rate of 11 g/min were constant for each experiment. All extrusion was conducted with distilled water unless otherwise specified. Collected samples

were dried at 40 C until brittle. The TWPs and commercial TVP were ground to a fine powder in a Braun food grinder and mixed as slurries for analysis.

Soluble protein

The soluble protein for 3.85% (w/v) slurries of samples and the control were determined in four solvents: water, 0.5 N NaCl, 2% SDS, and 0.02% BME. The slurries were shaken for 1.5 h, centrifuged for 5 minutes at 5000 rpms, followed by 15 min at 9000 rpm to ensure precipitation of fine particles. The supernatant was assayed for protein content spectrophotometrically (Shimadzu Biospec-1601) using the BCA assay (Pierce Chem. Co., Rockford, IL).

Statistical Analysis

The differences and interactions (pH and soluble protein) of TWP samples were calculated using analysis of variance (ANOVA). Significance was determined using least significant difference (LSD) with $\alpha=0.05$.

2. Significant Conclusions:

Influence of Treatments on pH

Addition of HCl, NaOH, and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ during extrusion effected ($p<0.05$) the pH of the final products as shown in Table 1. No measurements were made of product pH during extrusion.

Protein Solubility

Figure 1 shows the protein solubility of TWPs adjusted with NaOH or HCl, and collected at product temperatures of 140 and 150 C. The protein solubility of TWP with added calcium and collected at 140 and 150 C are shown in Table 2.

The properties of solvents used to solvate protein in samples help explain the chemistry of the system. Protein solubility of TWP in water was the standard to which solubility in other solvents was compared. An increase in TWP solubility in 0.5 N NaCl indicates an increase in ionic bonding. SDS solubility measured the amount of noncovalent interactions in TWP. BME reduces disulfide bonds. Increased protein solubility in BME indicates an increase in the number of disulfide bonds present in the sample.

The addition of acid and base at the rate of 11ml/min during extrusion adjusted ($p<0.05$) the pH with in approximately one pH unit, which effected ($p<0.05$) the protein solubility of the samples. Generally, a 0.1 decrease in pH is observed with a 10 C increase at the temperatures tested. However, there was not a difference ($p<0.05$) in the protein solubility due to extrusion

temperature. Minor adjustments to whey protein pH can be made during extrusion by adding acidic or basic solutions.

Addition of HCl and NaOH resulted in a decrease ($p < 0.05$) in soluble protein, which may be the result of increased ionic interactions. Above pH 7 there was a dramatic increase in protein solubility in 0.02% BME, suggesting disulfide bonding increases at higher pH.

Addition of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ also changed ($p < 0.05$) the pH, which effected the protein solubility of TWP depending on extrusion temperature of the product. Decreased solubility in calcium extrudates was only observed at higher calcium levels. The lower solubility may have been due to increased ionic and disulfide interactions.

Decreased solubility was obtained by adjusting physiochemical parameters during TWP extrusion. This preliminary research favors development of TWP suitable for use in coarse-ground meat products.

3. Anticipated Problems/Delays:

None

Publications:

Theses:

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.

Presentations:

Patent/Invention Disclosures:

Technology Transfer Activities

For information on licensing contact:

Western Dairy Center

Project Report

Reporting Period July 1, 1997 — December 31, 1998

Principal Investigators: Dr. Bart Weimer

Co-Investigators:

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

Institution's Project #: 97083

Project Completion Date: 12-31-99

National Research Plan (1997): Priority: 5; Goal: 1; Tactic: 4;
Identify and pursue the health and nutritional benefits of milk; to leverage bioactive peptides in milk for positioning or potential positioning; investigate microbial enzymatic activities leading to the formation of bioactive compounds in milk.

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 *Lb. 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 have been screened for peptide production from alpha-casein 1-23, and found that *Lb. helveticus* CNRZ32 produces at least 2 proteases and that the peptide pattern is a product of those enzymes. Pure peptides with

bioactivity have been synthesized and were as standards for further analysis of proteolytic patterns. The gene sequence is partially determined, which will be combined with the hydrolysis patterns to form a classification system. Accumulation of peptides is being determined as the hydrolysis progresses.

Hydrolysis of the alpha-casein 1-23 fragment by lactobacilli was complete within 15 minutes of incubation at 37°C. Identity of the resulting peptides were determined for CNRZ32 and protease deficient mutants by HPLC/MS and co-elution of synthetic peptide standards. Multiple peaks were observed with 11 dominate peptides being produced in the wildtype. The most noteworthy is the 1-9 fragment that was produced at high levels in all strains tested. It appears that the substrate is hydrolyzed once in various locations that result in two fragments, opposed to hydrolysis of each fragment in multiple locations. For example, one fragment pair was 1-9 and 10-23. In some cases, one part of the pair was not observed. However, since every peak was not sequenced, it is possible that it was present and not identified. Alternatively, these peptides may have been metabolized by cultures during incubation, but this is unlikely considering the short incubation times used in the assay. These data are ready to be used to define a protease classification system.

1. Significant Progress against Objectives:

All objectives are being completed as listed in the proposal.

2. Significant Conclusions:

Numerous hydrolysis patterns are found in the strains. At least 2 proteases seems to be produced in *Lb. helveticus* CNRZ32. Confirmation of the gene sequence is underway, and a classification system is being developed based on both sets of information.

3. Anticipated Problems/Delays:

Due to personnel changes the classification system work has not begun

Publications:

One submitted

Theses:

none

Published Abstract:

none

Presentations:

none

Patent/Invention Disclosures:

none

Technology Transfer Activities

For information on licensing contact:

Visitors Hosted:

Western Dairy Center

Project Report

Reporting Period July 1, 1997 — December 31, 1998

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: 12-31-99

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 whole 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 were initiated. Preliminary data are encouraging for locating, cloning, and sequencing the gene.

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 may be active in Cheddar cheese curd.

3. Anticipated Problems/Delays:

none

Publications:

Dias, B., and B. C. Weimer. 1999. Production of volatile sulfur compounds in Cheddar cheese slurries. *Int. Dairy J.* (accepted).

Weimer, B., K. Seefeldt, and B. Dias. 1999. Sulfur metabolism in bacteria associated with cheese. *Antonie van Leeuwenhoek* (accepted).

Ummadi, 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).

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 flavor 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.
- 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.
- Broadbent, Jeffery R., Charlotte Brennand, Mark E. Johnson, James L. Steele, Marie Strickland, and Bart C. Weimer. 1997. Contributions by starter and selected 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. Aromatic amino acid catabolism by lactococci. *Le Lait* 77:371.
- Weimer, B. C., C. Brennand, J. Broadbent, J. Jaegi, M. Johnson, F. Milani, J. Steele, and D. 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

Published Abstract:

Presentations:

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 Management, Inc. Conference on Cheese Flavor. Chicago, IL.

Patent/Invention Disclosures:

Technology Transfer Activities

The a strain of the brevibacteria has been licensed to Gist-brocades Inc. A second strain has been provided to Rhodia Inc. for evaluation.

For information on licensing contact:

Bart Weimer or Carl Brothersen

Visitors Hosted:

Western Dairy Center

Project Report

Reporting Period July 1, 1997 — December 31, 1998

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: 12-31-99

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

Lactic acid and brevibacteria were screened for fatty acids produced from branch chain amino acids. Each strain produced various amounts of fatty acids from each amino acid, 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.

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. Further work is needed to define the mechanisms associated with the increases.

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 are being explored.

3. Anticipated Problems/Delays:

Due to personnel changes the project has been delayed in defining the observations in slurries.

Publications:

none

Theses:

Published Abstract:

none

Presentations:

none

Patent/Invention Disclosures:

Technology Transfer Activities

For information on licensing contact:
Bart Weimer (435) 797 3356

Visitors Hosted:
