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## 1994 Annual Report

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WESTERN CENTER FOR  
DAIRY PROTEIN RESEARCH  
AND TECHNOLOGY

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Researching the Western  
U.S. Dairy Industry's Future

ANNUAL REPORT  
FISCAL YEAR 1994

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## WCDPRT ACTIVITIES SUMMARY

The Western Center for Dairy Protein Research and Technology (WCDPRT) was very active during the 1994 fiscal year. The activities of the Center are listed below.

1. A new research area "Function of Proteins and Enzymes in Low-fat Cheeses" was approved in the Center Annual Plan.
2. Nineteen research projects were active during the year including seven projects in the new low-fat cheese area.
3. Nine research projects were completed.
4. Ten research projects will be continued into FY95
5. The Center Annual Meeting was held on July 28 and 29, 1994, at Sun Valley, Idaho. A large group representing both dairy producers, processors and researchers attended and provided significant input onto the future direction of the Center.
6. The Center Technical Advisory Committee met on July 28, 1994, and reviewed ten research proposals submitted to the Center for funding in FY95. Six of these proposals were recommended to the Operational Advisory Committee. This year the TAC was made up of three (3) dairy industry representatives, one (1) dairy researcher from Oregon State University, one (1) member of the National Dairy Board Staff, the Director and Assistant Director of the Center.
7. The Center Operational Advisory Committee met on July 29, 1994, and reviewed the operation of the Center. The OAC approved three research proposals for funding in FY95, and asked for revision of three additional proposals. The OAC heard reports from four ad hoc committees that met during the Annual Meeting.
8. The Center cosponsored two conferences to facilitate technology transfer. They were: Symposium on Ultra-high Temperature Processing of Milk, held at Utah State University; and Biogenic Amines in Foods and Beverages. held at Oregon State University.
9. With the approval of the Low-fat Cheese Project the Center a collaborative effort with researchers at the Wisconsin Center for Dairy Research and the Minnesota-South Dakota Dairy Foods Research Center. The meeting to plan and coordinate the research was held in Salt Lake City.

Investigators at Utah State University continue to administer a State of Utah Center of Excellence in Dairy Foods Technology. The combined resources of these Centers prove to be an Excellent leverage so that more research can be conducted, with results being transmitted to all supportive and contributing parties.

**WESTERN CENTER FOR DAIRY PROTEIN  
RESEARCH AND TECHNOLOGY**

**OPERATIONAL ADVISORY COMMITTEE**

Pursuant to the WDFRC proposal and contract with the National Dairy Promotion and Research Board, the voting members of the Operational Advisory Committee are:

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2111 Wilson Blvd., Suite 600  
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WESTERN CENTER FOR DAIRY PROTEIN  
RESEARCH AND TECHNOLOGY

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**WESTERN CENTER FOR DAIRY PROTEIN  
RESEARCH AND TECHNOLOGY**

**BUDGET REPORT  
FISCAL YEAR 1994**

NATIONAL DAIRY PROMOTION AND RESEARCH BOARD \$500,000

**REGIONAL/INDUSTRY SUPPORT:**

Utah Dairy Commission	\$50,000
United Dairymen of Idaho	\$50,000
Oregon Dairy Products Commission	\$40,000
Western Dairy Farmers' Promotion Association	\$10,000

Kraft General Foods, Inc.	\$5,000
Schreiber Foods, Inc.	\$5,000
Marschal-Rhone Poulenc, Inc.	\$5,000

State of Utah Center of Excellence "Center for Dairy Foods Technology	\$130,000
--	-----------

TOTAL REGIONAL/INDUSTRY SUPPORT \$295,000

**FY94 TOTAL DAIRY RESEARCH CONTRIBUTIONS \$795,000**

**FY93 BALANCE FORWARD \$283,059**

**TOTAL AVAILABLE FUNDS FOR FY91 RESEARCH \$1,078,059**

**FY94 COMMITTED RESEARCH FUNDS**

Western Dairy Foods Research Center	(\$754,064)
State of Utah Center of Excellence	(\$130,000)
Administrative	(\$60,000)

**TOTAL FY94 COMMITTED RESEARCH FUNDS \$944,064**

**FY94 BALANCE FORWARD \$133,995**

## Financial Summary of Approved Projects 1993-96

Project Title	FY93	FY94	FY95	FY96
Production of Extracellular Proteases of <i>Brevibacterium linens</i> for Use in Lowfat Cheese - Weimer, USU	\$35,625	\$39,293	0	0
Bacteriophage-Resistance Gene Replacement in <i>Lactococcus lactis</i> - Geller, OSU	40,860	41,571	44,228	0
Purification of Monospecific, Polyclonal Antibodies from Bovine Cheese Whey - Brown, USU	0	44,875	42,360	0
Rheology and Microstructure of Mozzarella Cheese - McMahon, USU	25,600	43,440	0	0
Function of Whey Proteins and Lactose in Age Gelation of UHT-Processed Milk Concentrate-Part 2-McMahon, USU	22,075	26,000	0	0
Extrusion Processing of Whey Proteins - Hansen, USU	47200	29650	0	0
Effects of Iron Fortification on Chemical, Physical, Microbiological and Nutritional Properties of Yogurt - McMahon, USU (Non-NDB funded)	26,700	29,000	0	0
Interactions Between Milk Proteins, Starter Cultures, and Hydrocolloidal Milk Fat Replacers - Weimer, USU	42,620	49,276	0	
Milk Protein Interactions and Gelation During Thermal Processing - Brown, USU	0	62,735	67,925	68,026
Using a Natural Nutrient Process to Improve Milk Quality and Extend Milk Shelf-Life Through the Reduction in Lipid Oxidation and Off-Flavors with Tocopherol (Vitamin E) Supplementation to Dairy Cows - Schelling, U. of Idaho	19,838	19,438	0	0
Influence of Preadsorbed Protein on Adhesion of <i>Listeria monocytogenes</i> to Dairy Food Contact Surfaces - Daeschel, OSU	28,399	29,544	0	0

**Financial Summary of Approved  
Projects 1993-96 (continued)**

<u>Project Title</u>	<u>FY93</u>	<u>FY94</u>	<u>FY95</u>	<u>FY96</u>
Using Whey for Improvement of Exposed Subsoils and Sodic and Saline-Sodic Soils - Hansen, USU	14,000	0	0	0
Growth of Bifidobacteria in Milk: Association with <i>Streptococcus thermophilus</i> and <i>Lactobacillus</i> Species and Measured by Genetic and Enzymatic Probes - Sandine, OSU	26,315	0	0	0
Development of High Protein Low-Fat Fermented Foods from Yogurt Cheese - Hansen, USU	0	39,242	0	0
Low-fat Cheese		300,000	300,000	300,000
<b>TOTAL</b>	<b>\$329,232</b>	<b>\$754,064</b>	<b>\$454,513</b>	<b>\$368,026</b>

**Project Title:** The Influence of Preadsorbed Protein on Adhesion of *Listeria monocytogenes* to Dairy Food Contact Surfaces

**Personnel:** M.A. Daeschel, Associate Professor, Department of Food Science and Technology, Oregon State University

J. McGuire, Associate Professor, Departments of Bioresource Engineering and Food Science & Technology, Oregon State University

H. Al-Makhlafi and C.K. Bower, Research Assistants, Department of Food Science and Technology, Oregon State University

**Funding:** Western Center for Dairy Protein Research and Technology, US Agency for International Development, Public Health Service Institutional Grant, and Oregon Agricultural Experiment Station

**Objectives:**

1. record b-lactoglobulin adsorption kinetic data on each of a series of silanized silicon surfaces that have been prepared to exhibit varying degrees of hydrophobic and hydrophilic character;
2. compare the measured adsorption kinetics to that predicted by a simple molecular model of interfacial behavior to obtain a measure of the rate at which conformational changes take place on each surface;
3. prepare adsorbed b-lactoglobulin layers of varying age on each type of surface, and quantify the adsorbed mass of each film;
4. expose the surfaces, with and without preadsorbed films, to *Listeria monocytogenes* in order to document the relationship between characteristics of each protein film and the extent and tenacity of resulting microbial adhesion; and
5. model the extent and tenacity of microbial adhesion as a function of contact surface hydrophobicity, or the nature of the preadsorbed film.

**Results:**

During this last year of the project (93-94) we focused on objectives 4 and 5. Project objectives 1 through 3 were completed during the first and second years of the project and are summarized as follows. The adsorption kinetics exhibited by a-lactalbumin (a-lac), b-casein, and bovine serum albumin (BSA) at hydrophilic and hydrophobic silicon surfaces were recorded with ellipsometry as well, and interpreted with reference to a simple mechanism for irreversible protein adsorption. These additional tests were performed in response to TAC recommendations, and constitute a natural extension of past work that involved measurement of b-lactoglobulin (b-lg) adsorption kinetics on silicon surfaces varying in wettability. With regard to b-lg, a model based on the mechanism described the data very well, enabling interpretation of the kinetic behavior in terms of contact surface hydrophobicity influences on rate constants affecting protein attachment and unfolding at the interface. In particular, both experimental and simulation results indicated that if the process of protein adsorption is resolved into two steps, the first being reversible adsorption defined by kinetic rate constants  $k_1$  and  $k_{-1}$  for attachment and detachment, the second being a conformational change defined by a kinetic rate constant  $s_1$  (resulting in conversion of reversibly adsorbed protein to an irreversibly adsorbed species),  $k_1$  and  $s_1$  increase with increasing solid surface hydrophobicity, while  $k_{-1}$  decreases. Quantitative consideration of possible mass transfer influences on the observed adsorption rates indicated that the experiments were not conducted in a transport-limited regime. In the

present work, a-lac, b-casein, and BSA adsorption kinetics were measured and interpreted with reference to the same model. To date, we have performed a number of kinetic experiments with each of the four proteins, but quantitative conclusions are still tentative. However, although molecularly dissimilar in several ways, differences in surface activity among these proteins at hydrophilic and hydrophobic interfaces can be at least qualitatively explained with reference to molecular flexibility and stability. These have been observed to be very important factors influencing a-lac, b-casein, b-Ig and BSA interfacial behavior at air-water interfaces, and a-lac, b-Ig and BSA interfacial behavior at solid-water interfaces. b-Casein has a largely unordered structure, but is distinctly amphiphilic; it adsorbed at a greater initial rate and in a greater amount than the other proteins on each type of surface. Results-to-date lead us to infer that it exhibits a higher affinity for hydrophilic surfaces. The globular proteins a-lac and BSA behaved in a manner more similar to that of b-Ig at each surface; however, the difference in a-lac adsorption to hydrophilic and hydrophobic surfaces was quite large, with a-lac adsorbing to a much greater extent on hydrophobic surfaces. a-Lac, a small and resilient protein, preliminarily appears to exhibit a higher initial adsorption rate, than does BSA, to hydrophobic surfaces as well. BSA consists of three large domains and nine subdomains. Its surface activity appears largely governed by relatively slow unfolding of one of these domains after adsorption. BSA did not appear to attain a plateau in adsorbed amount after eight hours of contact on either surface, although it did yield a greater adsorbed amount than a-lac in the same time period.

For objectives 4 and 5, experimental protocols were developed to establish consistent quantification of adhered bacterial cells (*L. monocytogenes*). Flow through chambers were employed for reproducible rinsing to establish baseline bacterial cell adhesion to surfaces. Computer driven microscopic Image analysis was used for visualizing and enumerating bacterial cells adhered to surfaces. Our results (In press, Appl. Environ. Micro.) clearly indicated significant differences in the numbers of adhered cells to surfaces with different pre-adsorbed proteins. Briefly, Protein films adsorbed onto surfaces were allowed to contact *L. monocytogenes* for 3 h. After an 8 h protein adsorption time, cell adhesion to surfaces coated with b-Ig was greatest followed by surfaces coated with b-casein and a-lactalbumin. Surfaces coated with BSA allowed the lowest adhesion.

The data obtained were interpreted with reference to a simple kinetic model for protein arrival and unfolding as well as molecular properties of each protein. The observed data were found to be consistent with the relative rankings of rate constants defining arrival and unfolding for these proteins.

### Impact of research to the Dairy Industry

The ability of proteins to adsorb to food contact surfaces is well recognized. It is possible to capitalize on this phenomenon by specifically allowing antimicrobial or otherwise surface-passivating proteins to adsorb and provide an active deterrent to bacterial adhesion. Antimicrobial proteins are particularly attractive to use because of their known chemical and physical properties, their record of safe and efficacious use and their demonstrated lethality toward *L. monocytogenes*. The wide spectrum of food contact surfaces present in commercial settings presents a challenge to development of generic strategies that prevent adhesion and biofilm formation. Our approach includes quantitative evaluation of protein surface behavior as a function of molecular properties and surface hydrophobicity. Past research supports the importance of both hydrophobic interaction and adsorption competition on formation of an interfacial film. This work will go a long way toward allowing us to optimize noncovalent immobilization of passivating components in order to provide an effective and easily implemented barrier to *Listeria* adhesion.

## Publications:

Al-Makhlafi, H., McGuire, J. and M.A. Daeschel. 1994. The influence of Preadsorbed Milk Proteins on the Adhesion of *Listeria monocytogenes* to hydrophobic and hydrophilic silica surfaces. Appl. Environ. Microbiology. ( In press)

Daeschel, M.A., McGuire, J. and Al-Makhlafi, H. 1992. Antimicrobial activity of nisin adsorbed to hydrophilic and hydrophobic surfaces. J. Food Prot., 55:731

Krisdhasima, V., McGuire, J. and Sproull, R. 1992. Surface hydrophobic influences on b-lactoglobulin adsorption kinetics. J. Colloid Interface Sci., 154:337

Suttiprasit, P., Krisdhasima, V. and McGuire, J. 1992. The surface activity of a-lactalbumin, b-lactoglobulin and bovine serum albumin I. Surface tension measurements with single component and mixed solutions. J. Colloid Interface Sci., 154:316

Suttiprasit, P. and J. McGuire. 1992. The surface activity of a-lactalbumin, b-lactoglobulin and bovine serum albumin II. Some molecular influences on adsorption to hydrophilic and hydrophobic silicon surfaces. J. Colloid Interface Sci., 154:327

**Project Title:** Using a Natural Nutrient Process to Improve Milk Quality and Extend Milk Shelf-Life Through the Reduction in Lipid Oxidation and Off-Flavors with Tocopherol (Vitamin E) Supplementation to Dairy Cows

**Personnel:** Gerald T. Schelling, Department of Animal and Veterinary Science, University of Idaho

Richard A. Roeder, Department of Animal and Veterinary Science, University of Idaho

John Montoure, Department of Food Science and Toxicology, University of Idaho

**Funding:** Western Center for Dairy Protein Research and Technology

**Introduction:**

The oxidation of milk causing off-flavors continues to be a problem in the dairy industry. A recent study of retail milk indicated that up to 38 % of the samples had unacceptable flavor. The role of vitamin E as an antioxidant in milk is recognized. However it is not currently clear what nutritional conditions of dairy cows are important for optimal vitamin E impact upon the milk tocopherol content, and therefore oxidation, of milk. This work is designed to provide information on vitamin E relative to lipid oxidation in milk.

**Objectives:**

The specific objectives of the study are as follows:

1. To evaluate the effect of various chemical and/or administration forms of vitamin E on milk vitamin E content and milk oxidation.
2. To determine the efficiency of gastrointestinal tract absorption and mammary transfer of vitamin E to milk.
3. To determine the effect of higher dietary levels of vitamin A on the vitamin E content of milk and milk oxidation.

**Results:**

To achieve objectives 1 and 2 of this research, an incomplete Latin square design with two periods and 16 lactating Holstein cows were used to evaluate four treatments. The treatments were control (C), 4,000 IU of oral dl-alpha-tocopheryl acetate (ODLT), 4,000 IU of oral micellized d-alpha-tocopherol (ODT), and 4,000 IU of injectable d-alpha-tocopherol (IDT).



Each vitamin E source was administered once as a single dose. The oral treatments were each given as a bolus in a gelatin capsule, and the injectable treatment was delivered into the muscle at two sites with a 3.8 cm needle. Total alpha-tocopherol secretions in milk due to treatments ODLT, ODT and IDT were 13.1, 27.2 and 109.6 mg. The total alpha-tocopherol milk secretion response to the IDT treatment ( $P < .0046$ ) was 3.01 % of the administered dose. The alpha-tocopherol milk secretions due to the oral treatments ODLT and ODT represented .36 and .75 % of the administered doses, with ODT being greater ( $P < .0028$ ) than ODLT.

The rate of milk oxidation was measured over an appropriate time period on milk samples with added ferric sulfate by using the thiobarbituric acid (TBA) procedure. Table 1 shows the decreased oxidation of milk as milk alpha-tocopherol is increased via more available vitamin E forms or administration modes. Relative milk oxidation was significantly ( $P < .01$ ) reduced from 100 % for the control (C) to 82.1, 77.8 and 68.6 % for the ODLT, ODT and IDT treatments respectively. Table 1 also shows the close relationship between blood alpha-tocopherol concentration and milk oxidation.

Table 1.  
Effect of Dietary Vitamin E Sources on Vitamin E Utilization and Milk Oxidation

Vitamin E Source	Blood Vitamin E	Milk Vitamin E	Milk Oxidation
	ug/ml	ug/ml	relative %
C	8.6	.64	100
ODLT	10.2	.72	82
ODT	11.3	.98	78
IDT	23.5	1.73	69

The effect of various levels of dietary vitamin A on milk vitamin E and milk oxidation was studied in an incomplete Latin square design with two periods using 16 mid-lactation Holstein cows to evaluate four treatments. All Cows received 4,000 IU of supplemental dl-alpha-tocopheryl acetate and graded supplemental vitamin A levels of 25,000 (low), 75,000 (medium), 225,000 (high) or 675,000 (very high) IU of vitamin A acetate per head per day. Increasing the dietary vitamin A levels decreased the vitamin E content of both blood and milk. Figure 2 shows the increase in milk oxidation commensurate with the decrease in vitamin E content of the milk caused by high dietary vitamin A levels. When the low vitamin A treatment was set at a relative oxidation of 100 %, the relative oxidation was 125, 119 and 155 % for the medium, high and very high vitamin A treatments respectively.

Table 2.  
Effect of Dietary Vitamin A Levels on Vitamin E Utilization and Milk Oxidation

Vitamin A Treatment	Blood Vitamin E	Milk Vitamin E	Milk Oxidation
IU/day	ug/ml	ug/ml	relative %
25,000	12.4	1.36	100
75,000	12.2	1.31	125
225,000	11.8	1.32	119
675,000	10.1	1.08	155

**Impact of Research:**

The results of these studies will allow for the more knowledgeable formulation of dairy rations to reduce milk oxidation. The impact on formulating dairy rations will be as follows:

1. Relative efficiency data of different chemical forms and/or administration routes will allow for the selection of the most cost effective means of providing vitamin E to achieve certain milk concentrations of vitamin E.
2. The negative impact of high vitamin A levels on vitamin E utilization provides the information needed to make the necessary dietary vitamin E adjustment to maintain certain milk concentrations of vitamin E.

**Publications:**

Pumphrey, W. M., K. G. DeYonge, J. A. Slusark, M. J. Garber, R. A. Roeder, W. K. Sanchez and G. T. Schelling. 1993. Absorption of various forms of vitamin E from the gastrointestinal tract and transfer across the mammary gland in lactating dairy cows. *J. Anim. Sci* 71:256, Suppl.1.

Schelling, G. T., R. A. Roeder, M. J. Garber, W. M. Pumphrey and W. K. Sanchez. 1993. The need for increased antioxidant capacity for optimal meat and milk quality. *Proc. Fifteenth International Congress of Nutrition Abstracts* 1:70. Adelaide, Australia.

Garber, M. J., R. A. Roeder, J. C. Miller, R. P. Richard and G. T. Schelling. 1993. Dose-response effects of vitamin E supplementation on carcass characteristics and storage properties of beef from dairy steers. *J. Anim. Sci.* 71:152, Suppl. 1.

Schelling, G. T., R. A. Roeder, M. J. Garber and W. M. Pumphrey. 1994. Bioavailability and interaction of vitamin A and vitamin E in ruminants. *J. Nutr.* (in press).

**Project Title:**                    **Function of whey proteins and lactose in age gelation of ultra-high temperature sterilized milk concentrate, Part 2.**

**Personnel:**                    Donald J. McMahon  
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Nutrition & Food Sciences Dept., Utah State University

**Funding:**                    WESTERN CENTER FOR DAIRY PROTEIN  
RESEARCH AND TECHNOLOGY  
Utah Agricultural Experiment Station  
USDA Agricultural Research Service

**Objectives:**

The mechanism by which age gelation in UHT sterilized milk concentrates occurs is still unknown. There have been many factors implicated and at best an empirical approach is taken to extend shelf life of sterilized milk products. UHT sterilization promotes association between  $\kappa$ -casein and  $\beta$ -lactoglobulin. In milk concentrates the concentration of whey proteins and lactose are increased. Their role in the age gelation process will be studied in this project. The specific objectives of this project are to:

1. Determine the influence of lactose concentration of milk concentrates on age gelation.
2. Determine the fate of  $\beta$ -lactoglobulin during storage of UHT sterilized milk concentrates.
3. Monitor changes in casein micelle structure during storage of UHT sterilized milk concentrates.

**Results:**

*Objective 1.*                    This was completed in part 1 of this project.

*Objective 2 & 3.*

Using antibodies to  $\beta$ -lactoglobulin,  $\alpha$ -lactalbumin,  $\alpha_{s1}$ -casein,  $\alpha_{s2}$ -casein,  $\beta$ -casein and  $\kappa$ -casein (obtained from J.J. Stastny, University of Illinois College of Medicine), goat anti-mouse IgG conjugated to 10 nm gold, a protocol for immunolabeling of milk samples was established and applied to milk, milk concentrated by ultrafiltration to 3X (volume reduction), and the UF milk heated to 110, 120, 130, and 140°C. The location of these proteins was determined (with the exception of  $\alpha_{s2}$ -casein which did not label). These proteins were then tracked during 12 months storage of the UHT (140°C) milk. A mechanism for age gelation in concentrated milk is proposed.

### **Abstract 1.**

A simple apparatus has been developed for a new "microcube" encapsulation of fluid milk samples in their prefixation preparation for electron microscopy. The new technique is based on making cubic wells in an agar gel layer, filling them with fluid milk samples, and sealing them with another agar gel layer. The individual wells are then separated by cutting from the initial block, providing .5 mm walls around the samples. The embedded material (milk, buttermilk, yogurt, etc.) is fixed, dehydrated, and embedded in a resin for transmission electron microscopy. The procedure is simpler, more versatile, reliable, and reproducible than other encapsulation methods used to prepare similar food samples. Agar gel tubes used in the other methods have several disadvantages such as the need for manual dexterity of the experimenter to make them, and difficulty in sealing the filled capsules properly. Results obtained by the microcube procedure were compared with results obtained by two methods using agar gel tubes and also by mixing a warm agar sol with fluid food samples. This latter method is simpler than agar encapsulation, but shows agar strands in the micrographs of the milk samples. This is particularly undesirable when investigating, for example, intermicellar strands of gelled UHT-sterilized milk concentrates. Microcube encapsulation produces superior quality images of the fluid food structure.

### **Abstract 2.**

A protocol has been established for aldehyde fixation and immunogold labeling of  $\beta$ -lactoglobulin in milk samples, using trichloroacetic acid precipitated milk whey protein from direct ultra-high temperature-sterilized milk retentate (ultrafiltration concentrated 3x by volume reduction). Microcube prefixation encapsulation was used for all samples. Aldehyde degradation of antigen was investigated using enzyme-linked immunosorbent assay (ELISA). Antigenicity of the samples is reduced by both aldehyde fixatives, but paraformaldehyde is less deleterious than glutaraldehyde. Paraformaldehyde (4%) with fixation times of 2 h, 3 h, 4 h, and 5 h and glutaraldehyde (1.5%) with fixation times of .5 h, 1 h, 2 h, and 3 h were investigated to determine the optimal fixation time for maximum protein antigenicity. The 4 h fixation with paraformaldehyde (4%) and 1 h fixation with glutaraldehyde (1.5%) best preserves antigenicity while providing adequate fixation of the protein. The paraformaldehyde fixation results in better sections at labeling. LR White resin polymerized at 50°C was satisfactory for the embedding of samples. Teleosteam fish gelatin (.1%) with normal goat serum (.1%) in 20 mM Tris buffer without bovine serum albumin proved to be an adequate blocking agent. The protocol localizes  $\beta$ -lactoglobulin in gelled and fresh UHT-sterilized UF milk retentate.

### **Abstract 3.**

Samples of anti- $\beta$ -lactoglobulin and anti- $\alpha$ -lactalbumin are used in Western blot analyses and immunolocalization studies of whey at various stages of preparation from fresh samples to ultra-high temperature-sterilized samples. Western blot analyses of the samples show that the antibodies are specific for the native as well as the complexed forms of  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin. Immunolocalization studies show that the native form of the whey proteins is more susceptible than the complexed form to leaching through the fixation, dehydration, and embedding processes of electron microscopy preparation. Most of the labeling observed in immunolocalization analyses of milk samples for  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin, identifies these proteins complexed with each other or with other milk proteins.

### **Abstract 4.**

Immunolocalization techniques are employed to elucidate the positions of  $\beta$ -lactoglobulin,  $\alpha$ -lactalbumin,  $\alpha_{S1}$ -casein,  $\alpha_{S2}$ -casein,  $\beta$ -casein, and  $\kappa$ -casein in milk at various stages of treatment. These treatments comprise fresh whole milk, skim milk, pasteurized milk, ultrafiltered milk, and direct and indirect sterilized (110, 120, 130, and 140°C) milk.

Heating of milk through pasteurization and UHT sterilization affects the distribution and alters the conformational state of some milk proteins. This is more pronounced with  $\beta$ -lactoglobulin where interaction with whey and micellar casein protein is observed as a function of processing temperature.  $\alpha$ -Lactalbumin and  $\kappa$ -casein show a weaker response.  $\alpha_{S1}$ -Casein and  $\beta$ -casein show heavy specific labeling concentrated on the micelles, but no effect of heating on protein distribution is evident with these proteins.  $\alpha_{S2}$ -Casein did not respond to these immunolocalization procedures.

### **Abstract 5.**

Immunolocalization techniques are employed to elucidate the positions of  $\beta$ -lactoglobulin,  $\alpha_{S1}$ -casein,  $\beta$ -casein, and  $\kappa$ -casein in stored UHT-sterilized UF milk retentate from the day of preparation through to age gelation. The milk retentate had been stored at room temperature and sampling done bimonthly. Denatured  $\beta$ -lactoglobulin complexes on the micellar surface during UHT preparation and moves back to the intermicellar spaces during prolonged storage. The labeling of  $\kappa$ -casein is negligible just after UHT preparation, but begins to increase with storage time forming linear patterns within the intermicellar matrix.  $\alpha_{S1}$ -Casein and  $\beta$ -casein label heavily throughout the experiment. Labeling for these two casein moieties appear very specific for the micelles

just after UHT preparation until ~10 months of storage. At this time the labeling spills into the intermicellar matrix, but still shows high specificity for portions of the micelles. Much of the structural integrity of the micelle is retained in the coagulum. Labeling for  $\alpha$ -lactalbumin and  $\alpha_{s2}$ -casein was slight and indeterminate.

A mechanism of age gelation of UHT-sterilized UF-concentrated milk is proposed. The loss of the  $\beta$ -lactoglobulin- $\kappa$ -casein complex from the micelles to the serum exposes the calcium-insoluble micellar  $\alpha_{s1}$ -casein and  $\beta$ -casein to the serum calcium. This would reduce micelle stability and promote coalescence of the micellar proteins, leading to coagulation. The tendrillar appendages appear to be the  $\beta$ -lactoglobulin- $\kappa$ -casein complex which entraps the micellar residues at gelation.

#### **Impact of Research:**

A mechanism for the understanding of age gelation in concentrated milk has been proposed. This may allow those in the dairy industry who plan to produce such a product to know the limitations of the process and how it can be optimized.

#### **Publications:**

Alleyne, M.C., McMahon, D.J., Youssef, N.N. and S. Hekmat. 1993. An apparatus for a new microcube encapsulation of fluid milk in preparation for transmission electron microscopy. *Food Struct.* 12:21-30.

Alleyne, M.C., D.J. McMahon, W.R. McManus, and L.C. Burgess. 1994. Characterization of immunolocalization of  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin in milk and whey samples. *Food Struct.* (Submitted for publication).

Alleyne, M.C., D.J. McMahon and W.R. McManus. 1994. Comparing antigenicity of  $\beta$ -lactoglobulin in UHT-sterilized milk retentate and whey using different modes of aldehyde fixation. *Food Struct.* (Submitted for publication).

Alleyne, M.C. and D.J. McMahon. 1994. Immunolocalization of caseins and whey proteins in milk at various stages of treatment from fresh whole milk to ultra-high temperature-sterilized ultrafiltered (3X) milk concentrate. *J. Dairy Science* (Submitted for publication).

Alleyne, M.C. and D.J. McMahon. 1994. Immunolocalization of caseins and whey proteins in ultra-high temperature-sterilized ultrafiltered (3X) milk concentrate stored at room temperature until gelation. *J. Dairy Science* (In preparation).

**Project Title:** Effects of Iron Fortification on Chemical, Physical and Microbiological and Nutritional Properties of Yogurt

**Personnel:** Donald J. McMahon  
Sharareh Hekmat  
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**Funding:** WESTERN CENTER FOR DAIRY PROTEIN  
RESEARCH AND TECHNOLOGY  
Utah Agricultural Experiment Station

**Objectives:**

- A. Produce low fat and non fat iron-fortified yogurt using FeCl<sub>3</sub>, Fe-Casein and Fe-Whey Protein complexes as the iron sources.
- B. Determine growth and viability of *Lactobacillus delbruekii* ssp. *bulgaricus* and *Streptococcus thermophilus* in iron-fortified yogurt.
- C. Determine iron binding sites using Electron Spectroscopy Imaging.
- D. Determine the best procedure for making high-quality iron fortified yogurt.

**Results:**

**Effect of iron fortification of yogurt on its manufacture, growth of lactic acid and spoilage bacteria, and lipid oxidation.**

Low-fat (2%) and non-fat iron fortified yogurt were made with three sources of iron, FeCl<sub>3</sub>, Fe complexed with casein, and Fe complexed with whey protein, at three levels (10, 20, 40 mg/kg). The ferrozine assay and thiobarbituric acid test were used to quantitate iron content and lipid oxidation respectively over one month of storage of the yogurt at 4°C. Survival of *Lactobacillus delbruekii* ssp. *bulgaricus* and *Streptococcus thermophilus* were monitored using MRS (pH 5.4) and M17 media respectively. To determine whether presence of iron would promote growth of pathogenic or spoilage microorganisms, regular skim yogurt mix and iron fortified yogurt mix were inoculated with *Escherichia coli* DH5a and *Pseudomonas fluorescens* (ATCC 31732) to 10<sup>3</sup> and 10<sup>5</sup> cfu/ml of yogurt.

Iron fortification had no effect on the rate of fermentation by the lactic cultures. After fermentation the lactic acid bacterial counts were 10<sup>8</sup> cfu/ml for both *L. delbruekii* ssp. *bulgaricus* and *S. thermophilus* and these decreased only slightly during one month of

storage. The bacterial counts for *P. fluorescens* and *E. coli* decreased to less than  $10^1$  cfu/ml at day 1 and 15 respectively. There were no significant increases in oxidation levels between iron fortified yogurt and non-fortified yogurt ( $P=.05$ ). No differences in flavor between the iron-fortified yogurt and non-fortified yogurt were detected in sensory analysis using an untrained panel and all samples were strongly liked. Our study shows that yogurt is a suitable vehicle for delivering iron to consumers.

#### **Binding of iron to casein and whey proteins and in Fe-fortified yogurt.**

Iron binding affinity of casein and whey protein was studied by fortifying skim milk with 10 mg Fe/100 ml of milk and adjusting its pH to 6.7, 6.2, 5.8, 5.3, 4.5, and 4.0. These samples were ultracentrifuged and casein pellets and whey solutions were collected, digested with nitric acid, and then analyzed for iron by inductively coupled plasma (ICP) spectroscopy. SDS-PAGE was used to determine whey protein composition over this pH range. To further study iron binding of casein at low pH, skim yogurt was fortified with  $FeCl_3$ , Fe complexed with casein, and Fe complexed with whey protein. Yogurt was freeze-dried on carbon grids and then examined without heavy metal staining by transmission electron microscopy. Electron spectroscopic imaging (ESI) was then used to acquire elemental images and produce a map of Fe distribution in the samples.

As pH decreased, Fe binding capacity of casein increased while that of whey protein decreased. SDS-PAGE of whey showed clear bands for  $\alpha_s$ -casein, b-casein and k-casein at pH's 6.7, 6.2, 5.8 and 5.3. These bands were missing at pH's 4.5 and 4, indicating that at higher pH's, some casein was retained in the supernatant after ultracentrifuging. The Fe measured in the whey was most likely bound to this casein. Using ESI it was observed that when yogurt was fortified with Fe-casein, the Fe remained bound to the casein and was distributed throughout the micelles; with Fe-whey protein, it was distributed throughout the non-micellar portion of the yogurt; with fortification by  $FeCl_3$ , virtually all the Fe was observed to be bound preferentially to the casein and was located within the casein micelles.

#### **Impact of Research:**

Increasing the resolution available when using scanning electron microscopy to study the microstructure of mozzarella cheese will allow this technique to be used effectively in studying its microstructure. Developing a method to more appropriately measure stretch of melted mozzarella cheese will allow research results to be more directly applied to industry practices in the manufacture of mozzarella cheese for use on pizzas.



**Publications:**

Hekmat, S., Reddy, M.I., McManus, W.R., and D.J. McMahon. 1994. Binding of iron to casein and whey proteins and in Fe-fortified yogurt. 89th American Dairy Science Association Meeting, *J. Dairy Sci.* 77(Supp. 1):35

Hekmat, S., Wiemer, B., and D.J. McMahon. 1994. Effect of iron fortification of yogurt on its manufacture, growth of lactic acid and spoilage bacteria, and lipid oxidation. 89th American Dairy Science Association Meeting, *J. Dairy Sci.* 77(Supp. 1):19

**Project Title:** Interactions between milk proteins, starter cultures and hydrocolloidal milk fat replacers.

**Personnel:** Bart Weimer, Dept. of Nutrition and Food Sciences, Utah State University  
Donald McMahon, Dept. of Nutrition and Food Sciences, Utah State University  
Mike LeFevre, initially Utah State University, currently Kerry Foods  
Chris Allyne, Dept. of Nutrition and Food Sciences, Utah State University  
Christina Beer, Dept. of Nutrition and Food Sciences, Utah State University

## **Introduction**

Health experts are urging people to reduce the amount of fat consumed in their diet. Consumers are demanding more lowfat and non-fat food products. Many food ingredient companies have responded by developing fat substitutes. Simplese® (The NutraSweet Co.) and Stellar™ (Staley Manufacturing Co.) are just two of these. To replace milk fat, non-fat substances (such as starch or protein) should mimic milk fat globules. This means the starch or protein fat replacer should have a similar size distribution and be able to interact with milk proteins the way fat globules do. Different companies have developed fat replacers for segments of the food industry. Though some products do not target milk fat replacement, some companies claim their product can act as milk fat replacers in dairy products. The lowfat and non-fat food market is exploding. We will examine how fat replacers interact with milk proteins to mimic milk fat globules.

Addition of fat replacers to milk creates a new dimension to the microbiology of fermented dairy products. Interactions between fat replacers and starter cultures are unknown. Fat replacers offer starter bacteria new sources of growth nutrients in milk. Starter cultures' growth and acid production will be influenced by protein- and starch-based fat replacers. Functionality of fat replacers will be effected by the proteinases, peptidases and metabolic end-products of lactic acid bacteria. We will investigate the interaction between starter cultures and fat replacers and the effect of each during cheese making.

## **Objectives**

1. Examine coagulation, protein binding, and stability of milk proteins in the presence of fat replacers.
2. Stability of fat replacers in dairy systems during growth of lactic starter cultures.

## **Results**

Research to date has focused on objective 2. Experiments were begun for objective 1 when the Post-doctoral fellow left the project. Another Post-doctoral fellow was found recently and work to complete objective 1 is underway.

Forty two starter cultures were screened for their ability to produce acid in milk and media containing different fat replacers which demonstrated starter cultures can degrade fat replacers to produce acid during growth. Data suggests acid production could be difficult to control with added fat replacer and is dependent on the strain and fat replacer used during manufacture as low levels of enzymes activity that degrade the fat replacers was found. Strain characterization was done using colorimetry for individual glucolytic, peptidolytic enzymes, and the Biolog system as a method to predict the rate at which strains degrade fat replacers. The Biolog system was successfully used to screen strains for glucolytic enzymes, but did not reveal the rate of degradation. Colorimetric assays for these enzymes indicated the rate of hydrolysis of starch-based fat replacers is relatively low but is a significant source of acid. Hydrolysis of protein-based fat replacers was less common in the starters tested, but if present significantly increases acid production and cell growth in milk. Slendid was the only fat replacer tested that was not hydrolyzed and inhibited growth of starter cultures.

Coagulation characteristics of milk containing fat replacers at various levels varied with each fat replacer added and is currently being evaluated. Preliminary results indicate bacteria are associated with the fat replacers in cheese. These data taken together suggest that cheese made with fat replacers be significantly different from the full fat version and the acid production rate during manufacture will be a source of variation. Distribution of the fat replacers also vary according to the type of replacer used.

### **Significance to the Dairy Industry**

Use of fat replacers is increasing in the dairy industry. Determination of the chemical interactions in cheese is required for their successful use in flavorful lower fat cheeses. This work highlights that protein-based fat replacer behave similarly, but different from starch-based fat replacers. Use of starch-based fat replacers tend to have more degradation due to microbial conversion of the sugars to lactic acid which impacts cheese making.

### **Publications**

none to date

### **Abstracts**

LeFevre, M. and B. Weimer. 1993. Metabolic characterization of low fat Cheddar cheese starter cultures. *J. Dairy Sci.* 76 (supp 1):130.

**Project Title:** Production of extracellular proteases of *Brevibacterium linens* for use in low-fat cheese

**Personnel:** Bart Weimer, Dept. of Nutrition and Food Sciences, Utah State University  
Ummadi Madhavi, Dept. of Nutrition and Food Sciences, Utah State University  
Benjamin Dias, Dept. of Nutrition and Food Sciences, Utah State University

### Introduction

Consumers are demanding more lowfat and nonfat foods. Many food ingredient companies have responded by developing fat substitutes made from protein, starch, and pectin. The dairy industry has little experience using fat replacers in fermented milk products.

Addition of fat replacers creates a new dimension to the microbiology of fermented dairy products. Interactions between fat replacers and dairy cultures are unknown. Functionality of fat replacers will be effected by the proteinases, peptidases, and other metabolites the bacteria used during cheese making.

Lack of flavor development and hard body in reduced fat dairy products is a common problem. Highly proteolytic organisms, like *Brevibacterium linens*, have been used to accelerate the ripening of full fat cheese. They sometimes cause too much proteolysis which result in strong flavored and soft bodied full fat cheeses. Use of this organism in lowfat cheese may produce a product which has an acceptable level of flavor and body. We will investigate the use of a whey-based medium to produce enzymes that increase flavor development and body in Cheddar cheese made with fat replacers.

### Objectives

1. Develop a whey-based medium for the production of extracellular proteases by *Brevibacterium linens*.
2. Determine the interaction of the rate of proteolysis from proteases produced by *Brevibacterium linens* in the presence of fat replacers and coagulating enzymes in milk.
3. Develop processing parameters required to produce Cheddar cheese with acceptable flavor and body using milk fat replacers and proteases from *Brevibacterium linens*.

### Results

Significant effort has been placed on objective 3 while objectives 1 and 2 are currently underway. Proteolysis in cheese made with *B. linens* differs depending on the strain used in manufacture. Fat replacer degradation occurs for protein- and starch-based fat replacers in laboratory conditions. These data indicate that use of *B. linens* as a flavor adjunct is possible, but care must be used for off-flavor generation during aging. Biochemical characterization

supports the observations made for cheese with each strain tested exhibiting different glucolytic, proteolytic, peptidolytic, and lipolytic activity in culture.

Initial results indicate media significantly influences protease production and activity. Media formulations are currently being evaluated. Peptidase and lipase activity is also influenced in a strain specific manner. Studies to determine the optimum media formulation to enhance desirable traits and minimization of undesirable traits are underway.

### **Significance to the Dairy Industry**

Use of *B. linens* has been shown to be an acceptable flavor adjunct for use in low fat cheese. Determination of cheese making and growth conditions will lead to recommendations for cheese making to increase desirable flavors and minimization of undesirable off-flavors with and without fat replacers. Currently, work continues to define specific enzyme systems that need to be increased for enhance flavor in lower fat cheese.

### **Publications**

none to date

### **Abstracts**

Dias, B. and B. Weimer. 1993. Peptidase activity of adjunct lactobacilli used in the manufacture of low fat Cheddar cheese. *J. Dairy Sci.* 76 (supp 1):122.

Dias, B., U. Madhavi, and B. Weimer. 1994. Characterization and comparison of peptidase and lipase activity among *Lactococcus lactis* ssp. *cremoris*, *Lactobacillus helveticus*, and *Brevibacterium linens*. American Society for Microbiology Annual Meeting, Las Vegas.

**Title:** Bacteriophage-resistance gene replacement in *Lactococcus lactis*

**Personnel:** Bruce Geller  
Department of Microbiology  
Oregon State University

**Funding:** Western Center for Dairy Protein Research and Technology

#### Introduction

My lab has cloned and sequenced a gene from *Lactococcus lactis*, called pip, which is required for bacteriophage infection. We have tested approximately 15 phages that attack our test strain (*L. lactis* ssp. *lactis* C2), and all but one phage (sk1) is completely resistant to the strain with a mutated pip. In addition, we have evidence that pip is required for phage infection of other strains.

The project is beginning its third year, and is in the process of creating better phage-resistant strains than are currently available. This is being done by exchanging a mutated copy of pip with the normal copy of pip in the phage-sensitive strain. The methods we are using for genetic exchange will allow the introduction of the mutated pip into many different strains of *L. lactis*. This should make it easy to convert any existing strain to a phage-resistant strain by exchanging pip.

#### Specific Objectives

The primary objective of the proposed research is: To replace the wild-type chromosomal pip gene with a mutated copy, in order to create a stable, phage-resistant strain.

Strategically, the intermediate aims necessary to attain the primary objective are:

- a. To make in vitro mutations in the cloned gene required for phage infections.
- b. To insert the mutated copies of pip into the chromosome and exchange it with the wild-type gene.
- c. To test the different mutants for viability, phage resistance, and

growth characteristics favorable to cheese-making.

### Significance

Phage infection of cheese and other dairy fermentations is the most significant cause of ruined fermented milk products. Because nearly 30 % of the raw milk produced in the United States is used to make cheese, even a small percentage of the milk ruined by phage contamination represents a large quantity of lost milk, as well as lost profits and higher consumer costs. Often the product of failed cheese fermentations must be disposed of entirely, or sold at a small fraction of what high quality products command. Reduction or elimination of this problem would decrease the waste of milk, and through economic means, would increase the consumption by humans.

The proposed research would create new strains of lactic bacteria for starter cultures that are more phage-resistant than currently available. This strain improvement project differs significantly from those in the past, in that it proposes to use molecular genetic engineering techniques to introduce mutational changes not practically possible by conventional strain improvement technology. It must be emphasized that these genetically engineered strains would contain no foreign DNA, no additional DNA of any kind, and would be food-grade organisms according to current FDA rules.

The proposed research plans to take what we have learned from our basic research on phage infections and apply it to the development of new strains. The new strains will be tested for their growth characteristics necessary to make cheese. This testing includes the effects of the new strains on milk proteins, such as the casein proteolytic activities and coagulation times. In this way, the aims of the proposed research are very much in keeping with the focus of the Western Center for Dairy Protein Research and Technology.

### Results

Since our last report on January 17, 1993, we have attempted to replace pip in LM2301 with a transposon-mutated copy. We have screened hundreds of potential isolates, and found at least 10 that have the correct phenotype. Our putative pip-replaced strains are phage c2 resistant and phage sk1 sensitive, indicating that the strains are not contaminants, but derived from LM2301. However, genomic Southern blots from the putative pip-replaced strains did not confirm that pip was replaced. In fact, the Southern blots revealed a wild-type size pip. This is particularly difficult to reconcile, because the putative pip-replaced strains are erythromycin sensitive (this was the selectable marker on the replacement plasmid), and were never selected for phage resistance. While one might suspect a contaminant, this is inconsistent with the phage sk1 sensitivity of the isolates.

Since June 1, I have been working full time on this project, in order to resolve the conflicting data. At this time it is still unclear if the putative pip-replaced strains have a transposon-mutated copy of pip in the chromosome. I am currently developing a polymerase chain reaction-based assay to screen the

isolates, which will be much less labor intensive than genomic Southern blots.

#### Deliverables

For FY95 (07\01\94-06\30\95), the deliverables are:

1. A "food-grade", mutated copy of pip, that can be used to integrate into the chromosome of *Lactococcus lactis* and replace the wild-type copy of pip, without leaving any foreign DNA in the chromosome.
2. A strain with the "food-grade", mutated copy of pip inserted into the chromosome and substituted for the wild-type copy.
3. A test of the pip-substituted strain described above. This will include tests of phage resistance and other favorable cheese-making characteristics.



Project Title: Development of high protein low fat fermented dairy foods from yogurt-cheese.

Personnel: Conly L. Hansen, Professor, Dept. of Nutrition and Food Sciences, Utah State University

Yehia A. El-Samragy, Visiting Professor, Dept. of Nutrition and Food Sciences, Utah State University

Funding: Western Center for Dairy Protein Research and Technology and Utah Agricultural Experimental Station

#### Objectives:

The main objective of this research project is to develop a process to use yogurt-cheese manufactured from low fat milk retentate as the principal base ingredient to produce new fermented dairy foods. The new products will be characterized with a high protein and low fat content. The possibilities of the fortification of the new products with different additives such as flavors, fruits, vegetables, etc., will be emphasized. This will increase the consumer acceptance to this product and will meet a wide range of nutritional and organoleptic qualities being demanded by different types of consumers according to age, sex, health, and national origin.

1. Develop high protein, low fat, yogurt-cheese from skim milk retentate and do a sensory evaluation on the product.
2. Create new dairy products using yogurt-cheese as a dairy base ingredient and do a sensory evaluation on the new food products.
3. Evaluate the chemical, microbiological and sensory changes in the new dairy foods during storage.

#### Results:

Table 1 indicates the gross composition of soft cheese like product and Cheddar cheese like spread.

The firmness and water-holding capacity of soft cheese like product were close to that determined for Cheddar cheese like spread (data not shown). The total solids content was within a very narrow range of 21.81 to 21.84%, respectively (Table 1).

Some preliminary trials were carried out to organoleptically evaluate the new product in a plain form called yogurt cheese. Taste panelists seemed to get confused because they directed their minds to yogurt more than cheese or something in between yogurt and cheese. We have got a general comment that they want this product

in a flavored form more than in a plain form. This feed back made us change our way of presenting this product to call it soft cheese like product when made in a plain form. Also, we presented it a flavored form using natural Cheddar cheese flavor and a yellow food grade color and called it Cheddar cheese like spread.

Data presented in Tables 2 and 3 summarize the response of the panelists to the two forms of this new fermented dairy food. The scores of the panelists for soft cheese like product were in the positive side for appearance (74%), flavor (70%), richness (66%), spreadability (58%) and overall acceptability (72%) (Table 3). The scores for the Cheddar cheese like product were in the positive side also, for appearance (70%), richness (54%), spreadability (54%), flavor (56%) and overall acceptability (58%) (Table 3). The score for the overall acceptability of the Cheddar cheese like spread was less than the soft cheese like product. This might be explained from the fact that the American consumer used to have the Cheddar cheese in a hard form with flavor ranging from mild to extra sharp. Perhaps using real pieces of fruit or vegetables along with flavor will be more acceptable to the consumer.

#### Significance:

This project will develop a method to manufacture from ultrafiltered skim and/or low fat milk a "yogurt-cheese" with a high protein and non-fat or low fat content. The plain form of yogurt-cheese will be flavored and/or have fruits added to produce a new dairy food that can be eaten alone as a healthy dairy food or served in many ways such as a salad dressing. This product should be well received by consumers because it will meet a wide range of nutritional and organoleptic qualities being requested by consumers of all ages, health status, sex and cultures.

The overall results of this proposal will increase milk usage by opening new markets for dairy products with unique properties which will lead to increased profits for the dairy producer.

#### Deliverable

1. The plain form of this high protein, low fat fermented dairy product manufactured from ultrafiltered low fat milk can be used as a dairy ingredient.
2. It can be Cheddar cheese flavored as was done in the current work or have fruits, vegetables or spices added to produce other new dairy foods, as we are working on now.
3. These can be eaten alone or as healthy dairy foods or served in many ways such as a salad dressing.

We think that this product should be well received by consumers because it will meet a wide range of nutritional and organoleptic

qualities being requested by consumers of all ages, health status, sex and cultures.

**Publications:**

El-Samragy, Y. A. and C. L. Hansen. 1994.  
Sensory Characteristics, Physical Properties and Composition of a New, High Protein, Low Fat, Fermented Dairy Food.  
Abstract. Presented at the 89<sup>th</sup> Annual Meeting of the American Dairy Science Association (ADSA), Minneapolis, Minnesota, July 1994.

**Table 1: Gross composition of soft cheese like product and Cheddar cheese like spread.**

Item	Soft cheese like product	Cheddar spread like spread
Total solids(%)	21.81	21.84
Protein (%)	11.39	11.30
Fat (%)	3.45	3.53
Lactose (%)	4.64	4.72
Ash (%)	1.69	1.65
Salt (%)	0.64	0.64
Titratable acidity (%)	1.30	1.34

Table 2: Panelists score of sensory evaluation of soft cheese like product.

Property	Like very much		Like moderately		Like slightly		Dislike slightly		Dislike moderately		Dislike very much		Total	
	No	%	No	%	No	%	No	%	No	%	No	%	No	%
Appearance	1	2	17	34	19	38	12	24	1	2	0	0	50	100
Flavor	1	2	17	34	17	34	12	24	3	6	0	0	50	100
Richness	0	0	15	30	18	36	12	24	5	10	0	0	50	100
Spreadability	0	0	8	16	21	42	14	28	7	14	0	0	50	100
Overall acceptability	0	0	15	30	21	42	7	14	7	14	0	0	50	100

31 Table 3: Panelists score of sensory evaluation of Cheddar cheese like spread.

Property	Like very much		Like moderately		Like slightly		Dislike slightly		Dislike moderately		Dislike very much		Total	
	No	%	No	%	No	%	No	%	No	%	No	%	No	%
Appearance	6	12	20	40	14	28	8	16	2	4	0	0	0	100
Flavor	2	4	12	24	14	28	13	26	6	12	3	6	0	100
Richness	2	4	5	10	20	40	13	26	9	18	1	2	0	100
Spreadability	4	8	11	22	12	24	17	34	5	10	1	2	0	100
Overall acceptability	1	2	19	38	9	18	8	16	12	24	1	2	0	100

**Project Title:** Extrusion processing of milk proteins.

**Principal Investigator:** Conly Hansen

**Project Title:** Rheology and Microstructure of Mozzarella Cheese

**Personnel:** Donald J. McMahon  
Craig J. Oberg  
Robert Fife  
Nutrition & Food Sciences Dept., Utah State University  
William R. McManus  
Biology Dept., Utah State University

**Funding:** WESTERN CENTER FOR DAIRY PROTEIN  
RESEARCH AND TECHNOLOGY  
Utah Agricultural Experiment Station

**Objectives:**

It is well known that the stretch and melt characteristics of Mozzarella cheese are important aspects of its appeal to consumers. Although there have been a number of recent investigations of the factors that affect these properties, our knowledge of why Mozzarella cheese stretches and melts is still very limited.

An understanding of the process of cheese melting would enable us not only to control the production of conventional Mozzarella cheese but also develop new cheese products designed to satisfy consumer demands for lowfat cheeses. The objectives of this project are to:

- A. Develop rheological and electron microscopic techniques for the measurement the melting and stretching phenomena of cheese.
- B. Study the protein-fat interactions using the techniques developed above to determine how stretch and melt properties are related.

**Results:**

A metal impregnation technique using tannin-ferrocyanide-osmium tetroxide to impart thermal conductivity to samples destined for scanning electron microscopy was adapted for use with mozzarella cheese. Images were obtained at low and high magnifications having superior characteristics, with minimal edge effects, charging, thermal drift, and decoration. High magnification images were recorded with 3 nm resolution (which approaches the resolution limit of ultra-thin sectioned biological materials (2.5 nm). This combination of metal impregnation, ultra fine iridium metal coating and low voltage field emission SEM yielded images of cheese surpassing any previously published work.

Melt and stretch tests have been modified so that measurements can be made on mozzarella cheese containing less fat than part skim mozzarella cheese. It has also been shown that the state of moisture in mozzarella changes as fat is removed.

**Impact of Research:**

Increasing the resolution available when using scanning electron microscopy to study the microstructure of mozzarella cheese will allow this technique to be used effectively in studying its microstructure. Developing a method to more appropriately measure stretch of melted mozzarella cheese will allow research results to be more directly applied to industry practices in the manufacture of mozzarella cheese for use on pizzas.

**Publications:**

McManus, W., McMahon, D.J. and C.J. Oberg. 1993. High-resolution scanning electron microscopy of milk products: A new sample preparation procedure. *Food Struct.* 12:475-482.

Oberg, C.J., W. McManus, and D.J. McMahon. 1993. Microstructure of Mozzarella cheese during manufacture. *Food Structure* 12:251-258.

McMahon, D.J., Oberg C., and W. McManus. 1993. Functionality of Mozzarella cheese. *Australian J. Dairy Technol.* 48(2):99-104.

**Project Title:** Purification of monospecific polyclonal antibodies from bovine cheese whey

**Personnel:** Rodney J. Brown, Nutrition & Food Sciences Dept., Utah State University  
William H. Scouten, Chemistry & Biochemistry Dept., Utah State University  
Premysl Konecny, Biotechnology Center, Utah State University

**Funding:** Western Center for Dairy Protein Research and Technology

### **Objectives:**

The goals of this research are production of monospecific polyclonal antibodies in bovine milk, and purification of these antibodies from cheese whey. Specific research objectives of the project are:

1. Optimization of antibody titers in bovine milk by testing various immunization schemes.
2. Development of a purification method using affinity chromatography with immobilized antigen for purifying antibodies from cheese whey.

### **Results:**

#### a/ Purification of non-specific antibodies by thiophilic chromatography

In the first phase of the project we focused on purification of immunoglobulin G from cheese whey. We presumed that it would be necessary to perform a preliminary purification/concentration of the antibody fraction (mainly immunoglobulin G) from cheese whey prior to obtaining a specific antibody by immunoabsorbent chromatography with an immobilized antigen. This concentration step would allow a higher yield of the antibody of interest. It would also allow us to use the residual immunoglobulin fraction for other purposes after isolating the specific antibody. For example, whey immunoglobulins have been suggested as a prophylactic dietary supplement for newborn calves instead of colostrum, or colostrum-derived preparations enhanced in immunoglobulins.

We have developed a method for the purification of immunoglobulin G (IgG) from sweet cheese whey based on specific interactions with a thiophilic adsorbent. Thiophilic gel (T-gel), a resin of the structure matrix-O-CH<sub>2</sub>CH<sub>2</sub>SO<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>SCH<sub>2</sub>CH<sub>2</sub>OH, has proved to be a useful tool for selective purification of immunoglobulins from various sources, mainly mammalian sera and ascites fluid. The thiophilic interactions are promoted by salt and require presence of lyotropic (water-structure-forming) salts.



Among the salts tested, sodium sulfate most efficiently promoted IgG adsorption on T-gel; 94% of the IgG was bound to the gel at 0.5 M salt concentration, and the amount of IgG eluted reached 75% (Fig. 1). We demonstrated that 0.5 M sodium sulfate was the optimal concentration in thiophilic chromatography of bovine IgG (Fig. 2). The amount of IgG eluted from the T-gel after adsorption of the protein at 40 °C was comparable to that carried out at 20 °C (Fig. 3, Table I). The capacities of two commercially available T-gels for bovine IgG were determined (Table I). The highest eluted (static) capacity achieved was 19.0 mg IgG/ml gel. The purity of IgG prepared by thiophilic chromatography (Fig. 4) was assayed using sodium dodecylsulfate-polyacrylamide gel electrophoresis, radial immunodiffusion and immunoelectrophoresis (Fig. 5, 6).

One-step thiophilic chromatography of crude sweet whey yielded 74% pure immunoglobulin G, and the resulting immunoglobulin retains its antigenic activity, as demonstrated by radial immunodiffusion and immunoelectrophoresis. This method provides very good results compared to the use of highly selective Protein G, which yielded 81% pure immunoglobulin G. For more details see Fig. 1-6 at the end of this report, and the complete data in our article, *J. Chromatogr.*, 673, 45 (1994).

In summary, thiophilic gel, which is more stable, sturdier and less expensive than most of commercially available affinity chromatography materials, proved to be useful for the isolation of bovine immunoglobulin G, yielding protein with a purity comparable to that obtained by Protein G chromatography. The capacities of a randomly chosen commercially available T-gel were found to be reasonable for bovine IgG production. The procedure requires the addition of an appropriate salt (sodium sulfate, potassium sulfate) which can be subsequently removed by ultrafiltration of the IgG-free whey, and recovered from the ultrafiltrate by reverse osmosis and eventually reused or recycled. The volume of cheese whey processed might be reduced by prior ultrafiltration to as little as 10%, which would be a requirement for the use of thiophilic chromatography (batch or column mode) since it would enable one to keep the amount of salt at reasonably low levels. Thiophilic gel can be used at elevated temperatures, so that cooling whey to room temperature would not be required.

#### b/ Antibody production in the milk of dairy cows

Two groups of randomly selected Holstein cows in middle lactation stage (4-5 months after calving) were immunized, each group received a different type of antigen. Dinitrophenol-keyhole limpet hemocyanin (DNP-KLH) and mouse immunoglobulin G (IgG) were chosen as model antigens. DNP is known to be a strong immunogen and keyhole limpet hemocyanin is a preferred carrier due to its phylogenetic diversity from mammal proteins. The DNP-KLH conjugate was expected to be a very good model antigen, stimulating a strong immune response. The rationale

behind the choice of mouse IgG was the potential marketability of the anti-mouse IgG antibody isolated from whey. This antibody might be competitive with anti-mouse IgG antibodies currently being raised in rabbits or goats, having the advantage of being prepared without the necessity of drawing blood from the animals.

Dairy cows were immunized with these antigens as follows. The immunization protocol was submitted and passed a process of peer review and an approval (USU Institutional Animal Care and Use Committee) and was applied as stated.

#### Immunization protocol

animals: dairy cows (Holstein) in early/middle lactation (2 groups of 3, + 2 controls, for a total of 8 cows)

antigens: a. mouse immunoglobulin G  
b. dinitrophenol-keyhole limpet hemocyanin conjugate (DNP-KLH)

- concentration: 1 mg/ml, final concentration [after dilution with adjuvant 1:1]
- dose: 10 mg of antigen

administration: subcutaneously, 4 sites (4 x 2.5 ml), cervical region, per immunization period

adjuvant: aluminum hydroxide (Imject Alum, Pierce)

boosters: 2-3 boosters, 3 weeks apart, same dosage as the initial injection

test bleeds: 7-10 ml, from jugular vein

milk collection: weekly

(first test bleeds and milk collection: 7-10 days after the 1<sup>st</sup> booster)

negative controls: serum and milk from the animals on day 0 before immunization, and serum and milk from the nonimmunized cows

Note: a/ The number of animals in each group (3) takes into account the risk of insufficient individual immune response or the loss of an animal, but is not sufficient to provide statistical evaluation of the experimental data.

b/ The choice of adjuvant was limited by the undesirability of using Freund's adjuvant when possible. Application of Freund's adjuvant involves minor stress to the animal, and its use is not desirable with dairy cows. Aluminum hydroxide was chosen as an efficient substitute that provided an enhanced immune response without a stress/danger to animals associated with Freund's adjuvant.

c/ Doses of the immunogens were chosen at an intermediate level capable of producing a good immune response, but are not necessarily optimal. More extensive testing of various amounts of antigens would be necessary for optimization.

### Preparation of whey

Milk was processed into sweet whey as follows. Milk samples (4-6 °C) were centrifuged at 16,000 g for 15 min to separate milk fat. Skimmed milk was warmed in a waterbath to 31-32 °C, and rennet (single strength rennet, diluted 1/20 with water, 4 ml of solution / liter of milk) was added. The milk was then incubated at 31-32 °C for 30-45 min. The resulting gel was cut, and the curd separated from the whey by centrifugation (16,000 g x 15 min). Whey was collected, assayed for the presence of specific antibodies (anti-DNP, anti-mouse IgG), and stored frozen at -80 °C.

### Analysis of antibody titers

We developed an ELISA (Enzyme Linked Immunosorbent Assay) for monitoring titers of specific antibodies against DNP and mouse IgG in both serum and whey. Test bleeds served as necessary controls of the presence of these antibodies. DNP-human serum albumin (DNP-HSA) was used as an antigen in the assay instead of DNP-keyhole limpet hemocyanin due to better solubility of the former. This does not make any difference in assaying the anti-DNP antibody, since it would recognize the DNP group only, regardless of the carrier.

Polystyrene 96-well plates were coated with 100 µl of 0.01 mg/ml DNP-HSA (Calbiochem, LaJolla, CA) or 0.001 mg/ml mouse IgG (Rockland, Gilbertsville, PA) in 0.06 M carbonate-bicarbonate buffer, pH 9.6 by incubation at 4 °C overnight. After washing with phosphate buffered saline (PBS, 0.01M sodium phosphate, 0.15 M sodium chloride, pH 7.20), the plates were incubated with a blocking solution (0.1 g bovine serum albumin/ ml PBS) for 1 hour at 37 °C and washed again with PBS. Sera or whey samples diluted with PBS were added, and then the plates were incubated for 1 hour at 37 °C and washed with PBS. Horseradish peroxidase-anti-bovine IgG conjugate (Sigma, St. Louis, MO) diluted 1/25,000 in PBS was added and incubated for 1 hour at 37 °C. Excess conjugate was washed off with PBS, and the enzyme substrate 3,3',5,5'-tetramethylbenzidine (1-Step Turbo-TMB-ELISA, Pierce, Rockford, IL; 100 µl) was added, followed by 30 min incubation at room temperature. The reaction was stopped with 1 M sulfuric acid (100 µl) and absorbances measured at 410 nm in microplate reader (EL 311, Biotek Instr., Winooski, VT).

Absorbance readings were compared against those of negative controls, i.e. whey and sera samples from non-immunized cows, and whey and sera taken from the cows before immunization. Analysis of antibody titers was done weekly, starting 3rd week after the initial injection.

The ELISA does not allow determination of absolute concentration of anti-DNP or anti-mouse IgG antibodies since there is no standard for these antibodies currently available but it was possible to follow the increase in antibody levels in titers for serum or whey. The difference

between absorbances at 410 nm of the samples and blanks-controls indicating the end-point (end-titer) was set arbitrarily at 0.1 for anti-DNP antibody, and 0.05 for the anti-mouse IgG antibody.

#### Antibody response in serum and whey for DNP-KLH and mouse IgG

Enhanced levels of anti-DNP antibody were detected in blood sera and whey samples from cows No. 7604 and 7802 immunized with DNP-KLH after the second booster injection, i.e. after ca. 40-50 days after the initial injection (Fig. 7). These high antibody titers were observed between days 48-62, and then began decreasing. An increase in anti-DNP titers was observed in serum and whey from cow No. 7604 two weeks after the first booster injection, followed by a slight drop in the titers. Cow No. 7582 gave a very poor immune response throughout.

As expected, anti-DNP antibody titers are significantly lower in whey compared to serum, with a ratio of approximately 1/20-1/100.

One cow (No. 7782) of the group of three injected with mouse IgG gave a good immune response (Fig. 8). Anti-mouse IgG titers peaked two weeks after second and third boosters. There was a good correlation between increase of antibody titers in serum and whey during the immunization period. Anti-mouse IgG titers in whey were 1/10-1/80 lower than those in serum.

Cow No. 7266 died on day 64 from. Autopsy revealed that the cause of death was right site displaced abomasum, and it had no connection with the immunization conducted.

Larger amounts of whey (ca. 6-8 liter each) were prepared from milk from cows No. 7604, 7782, 7802 after antibody titers in serum and whey reached their peaks. The peak titers for these cows were:

cow No. 7604: day 55, anti-DNP titer 1/640,

cow No. 7802: day 55, anti-DNP titer 1/1280; day 62, anti DNP-titer 1/640,

cow No. 7782: day 83, anti-mouse IgG titer 1/320; day 90, anti-mouse IgG titer 1/160.

The whey was prepared as described above and kept frozen at -80 °C for use in immunoaffinity chromatography.

#### c/ Preparation of immunoaffinity adsorbents

We selected two methods of preparation of affinity adsorbents with an immobilized antigen. Among the wide selection of immobilized techniques and commercially available activated gels, tresyl chloride activated agarose and 3M Emphaze represent materials allowing preparation of immunoadsorbents with high binding capacity, low non-specific binding capacities, operating at high flow rates and having potential for regeneration and reuse.

Tresyl chloride (2,2,2-trifluoroethanesulfonyl chloride)-activated gel can be used to couple ligands possessing sulfhydryl and/or amine groups. The activated gel is suitable for immobilizing pH and temperature sensitive proteins, since it is very efficient at neutral pH and even 4 °C.

3M Emphaze Biosupport Medium AB 1 (Pierce, Rockford, IL) possesses an azlactone group, highly reactive with nucleophilic functional groups, typically amines and thiols. Proteins containing primary amines couple to the gel quickly at mild conditions (room temperature, pH 7.5-9.0, 1-2 hours). The character of the support (rigidity, porosity, chemical resistance) allows to use Emphaze in medium pressure, fast flow configurations. It can withstand pressure up to 100 psi and can be exposed to high flow rates (up to 3000 cm/hour) which makes it suitable for large scale applications with high throughputs. Emphaze has been reported by the manufacturer to be easily reused, and derivatized gel retained more than 95 % of its initial capacity over 130 cycles.

Immobilization of proteins of interest (mouse IgG, BSA) on Emphaze was conducted under conditions described. Protein (5 mg/ml in 0.1 M sodium borate buffer / 0.8 M sodium sulfate, pH 9.0) was added to dry gel (5 mg protein / 60 mg dry weight gel, i.e. 10 mg protein / ml wet gel). The coupling reaction proceeded at room temperature for

1 hour, then the gel was thoroughly washed with phosphate buffered saline (PBS; 0.15 M sodium chloride / 0.01 M sodium phosphate buffer, pH 7.20), and remaining unreacted azlactone groups were quenched with 1 M ethanolamine, pH 9.0 at room temperature for 2 hours. After thorough washing with 1.0 M sodium chloride / PBS, pH 7.20, and then PBS, the gel was analyzed for the amount of protein immobilized, using bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) under standard test conditions.

Mouse IgG was immobilized with 85-87% efficiency, resulting in 6.1-6.3 mg mouse IgG bound per 1 ml gel. Immobilization of BSA yielded a derivatized gel with 5.0-5.3 mg BSA / ml gel (63-66 % binding efficiency). The results indicate that using a higher protein/gel ratio in the reaction mixture may yield gels with 15-20 mg protein immobilized per 1 ml gel.

We immobilized BSA on Emphaze and tresyl chloride activated agarose, instead of DNP-HSA. We found out experimentally that the latter had no free amine groups available for binding on the activated gels, as a result of saturation of these groups with DNP. In the alternative approach we will immobilize BSA, HSA or rabbit serum albumin (RSA) on the gel, and then DNP groups will be introduced by reaction with the remaining amine groups of the proteins. It is likely that any of these serum albumins will be a suitable base for DNP groups, since the anti-DNP antibody would recognize only the DNP groups, not reacting with the protein.

#### **Interpretation of the significance of the results:**

a/ We have developed a selective method for purification of IgG from whey. This might be applied as a preliminary purification step prior to isolation of monospecific antibodies using

immunoaffinity chromatography. Alternatively, it might provide, in only one step, fairly pure immunoglobulin G. In either case, it represents a useful tool for antibody purification from whey. It might be employed in antibody production for both veterinary and human needs, and might ultimately lead to increasing the value of cheese whey.

b/ Immunization of dairy cows with two different antigens (dinitrophenol-keyhole limpet hemocyanine and mouse IgG) showed that specific polyclonal antibodies can be produced in milk (whey), which might give us the advantage of preparation of the antibodies without the necessity of drawing blood from the animals.

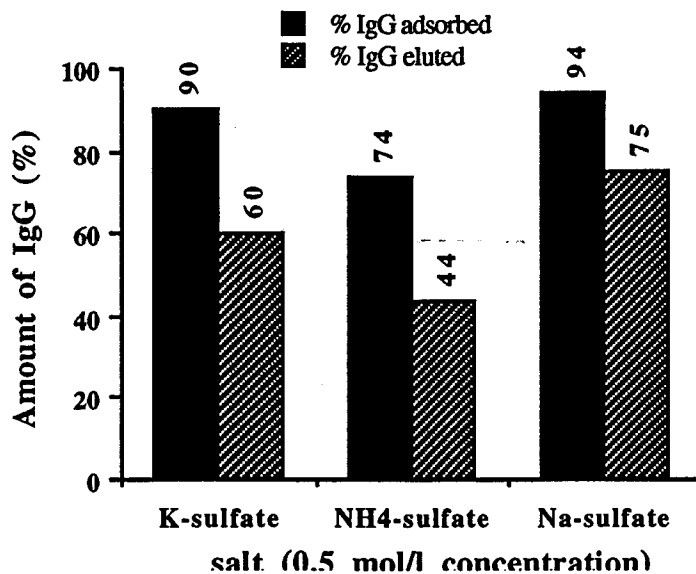
c/ Preparation of immunoabsorbents with immobilized antigens to purify antibodies has been shown to be feasible in preliminary experiments. Further work is required to optimize these procedures.

#### **Future plans:**

In the next part of the project we will focus on developing methods to immobilize DNP and mouse IgG to prepare immunoabsorbents that will be used to purify anti-DNP and anti-mouse IgG antibodies, and we will characterize these immunoabsorbents. We will develop conditions for purification of specific antibodies from whey, and analyze the purity and antigenicity of the final products.

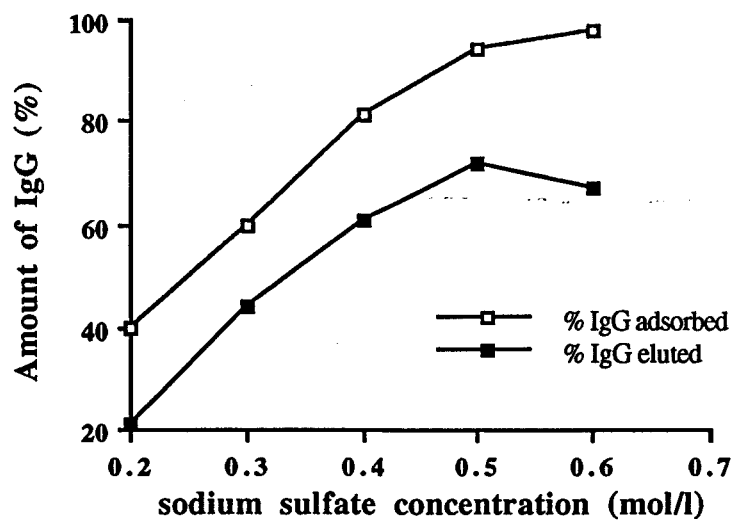
#### **Presentations, publications:**

A paper describing the method for the purification of IgG from whey using the thiophilic adsorbent has been published [P. Konecny, R.J. Brown, W.H. Scouten, Chromatographic Purification of Immunoglobulin G from Bovine Milk Whey, J. Chromatogr., 673, 45 (1994)]. These results were also presented at the 207th American Chemical Society National Meeting, Division of Biochemical Technology session in San Diego, CA in March 1994.



**Fig. 1:** Effect of salts on thiophilic adsorption of bovine IgG

Aliquots (0.5 g) of suction-dried T-gel (matrix: crosslinked 4 % beaded agarose) were equilibrated with 0.5 mol/l salt solutions in 0.05 mol/l sodium-potassium pH 7.5, then treated with 2 ml of IgG solutions (1 mg/ml) for 30 min. The gels were washed with the appropriate salt/buffer, then adsorbed IgG was eluted with 0.05 mol/l sodium-potassium phosphate buffer, pH 7.5. IgG concentration in both washing and elution fractions was quantified. The experiment was done in a batchwise mode. Values reported are the average of five determinations, the standard deviation being less than  $\pm 4\%$ .

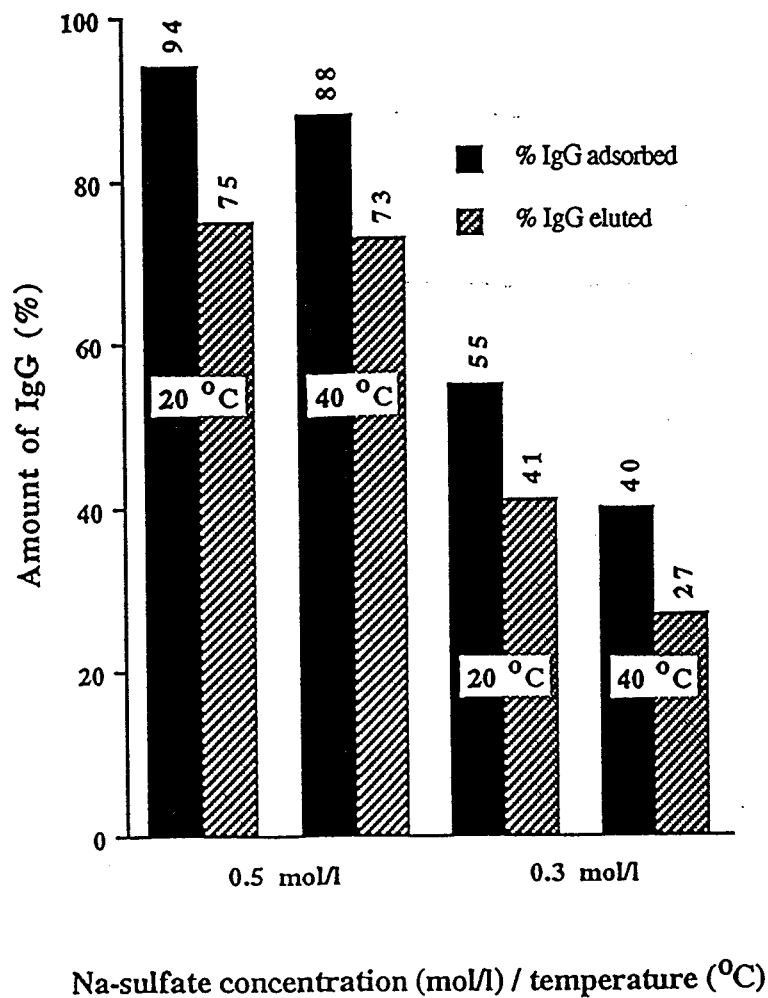


**Fig. 2:** Effect of salt concentration on thiophilic adsorption of bovine IgG

T-gel was equilibrated with sodium sulfate solutions of 0.2-0.6 mol/l concentration and then treated as described (Fig. 1). The amount of IgG in washing and elution fractions was determined for various salt concentrations. Values reported are the average of five determinations, the standard deviation being less than  $\pm 4\%$ .



## T-gel: Effect of temperature on adsorption of bovine IgG



**Fig. 3:** Effect of temperature on thiophilic adsorption of bovine IgG

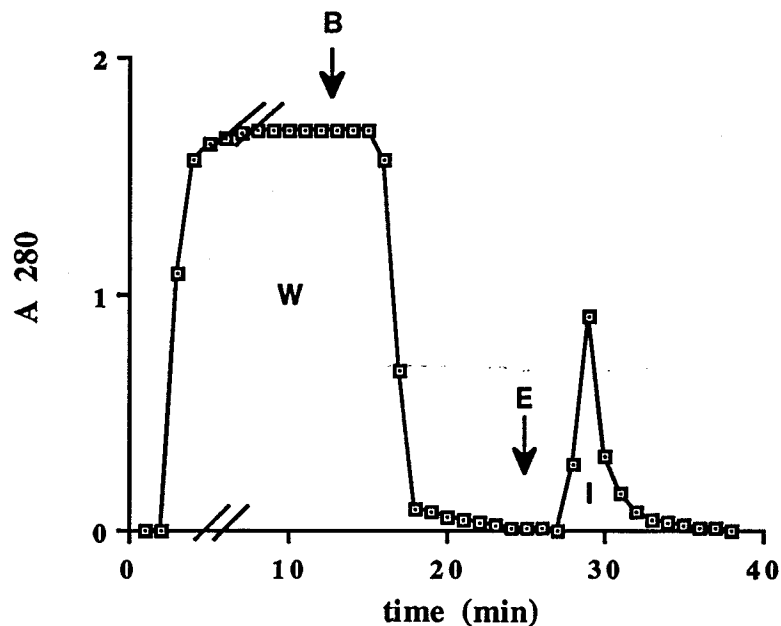
Adsorption of IgG to T-gel (0.5 g aliquots of suction-dried gel) equilibrated with sodium sulfate solutions of 0.3 and 0.5 mol/l concentrations (in 0.05 mol/l sodium-potassium phosphate buffer, pH 7.5) was performed at 20 °C and 40 °C, respectively. Elution was done as described (Fig. 1) at 20 °C and the concentration of IgG in all fractions was quantified spectrophotometrically. Values reported are the average of five determinations, the standard deviation being less than  $\pm 4\%$ .

Table I: Capacity of T-gels and Protein G- Sepharose 4FF for bovine IgG

	Dynamic capacity at 10% breakthrough, at a linear flow-rate of 34 ml/h (mg IgG/ml gel)	Eluted (static) capacity (mg IgG/ml gel)
T-gel (crosslinked 4% beaded agarose)	12.1± 0.3 <sup>a</sup>	12.0±0.4 <sup>a</sup>
T-gel (6% beaded agarose)	18.2±0.3 <sup>a</sup> 17.3±0.5 <sup>b</sup>	19.0±0.4 <sup>a</sup> 18.3±0.5 <sup>b</sup>
Protein G-Sepharose 4FF	22.1±0.4 <sup>a</sup>	19.0±0.6 <sup>a</sup>

<sup>a</sup> at 20 °C

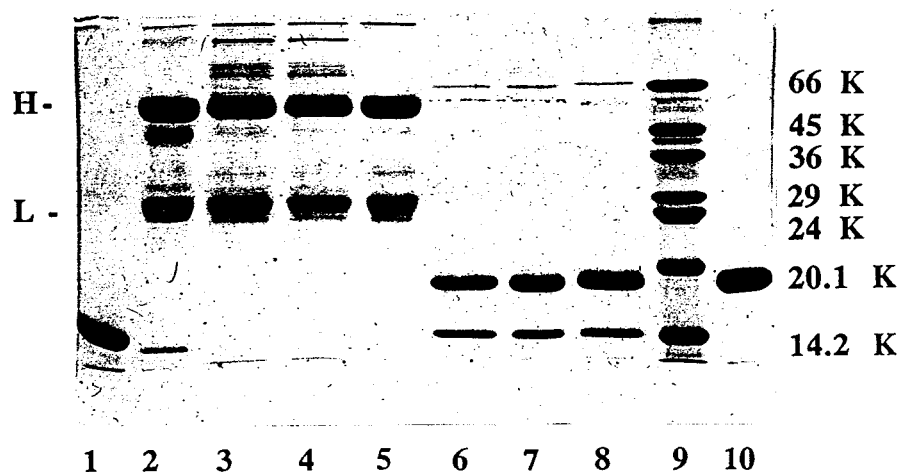
<sup>b</sup> at 40 °C



**Fig. 4:** Purification of IgG from whey by thiophilic chromatography

Clarified sweet whey (85 ml), with solid sodium sulfate added to concentration of 0.5 mol/l, was applied to a column (1.5 cm x 1.3 cm) of T-gel (matrix: 6 % beaded agarose) equilibrated with 0.5 mol/l sodium sulfate / 0.05 mol/l sodium-potassium phosphate, pH 7.5. After washing with the same buffer (B), adsorbed IgG was eluted with 0.05 mol/l sodium-potassium phosphate, pH 7.5 (E). The chromatography was performed at a flow-rate of 1.0 ml/min. Fractions from the chromatography (W: IgG-depleted whey, I: IgG fraction) were characterized by SDS-polyacrylamide electrophoresis (see Fig. 5).

**SDS-PAGE of fractions from chromatographic purifications  
of sweet whey on T-gel and Protein G - Sepharose 4FF**

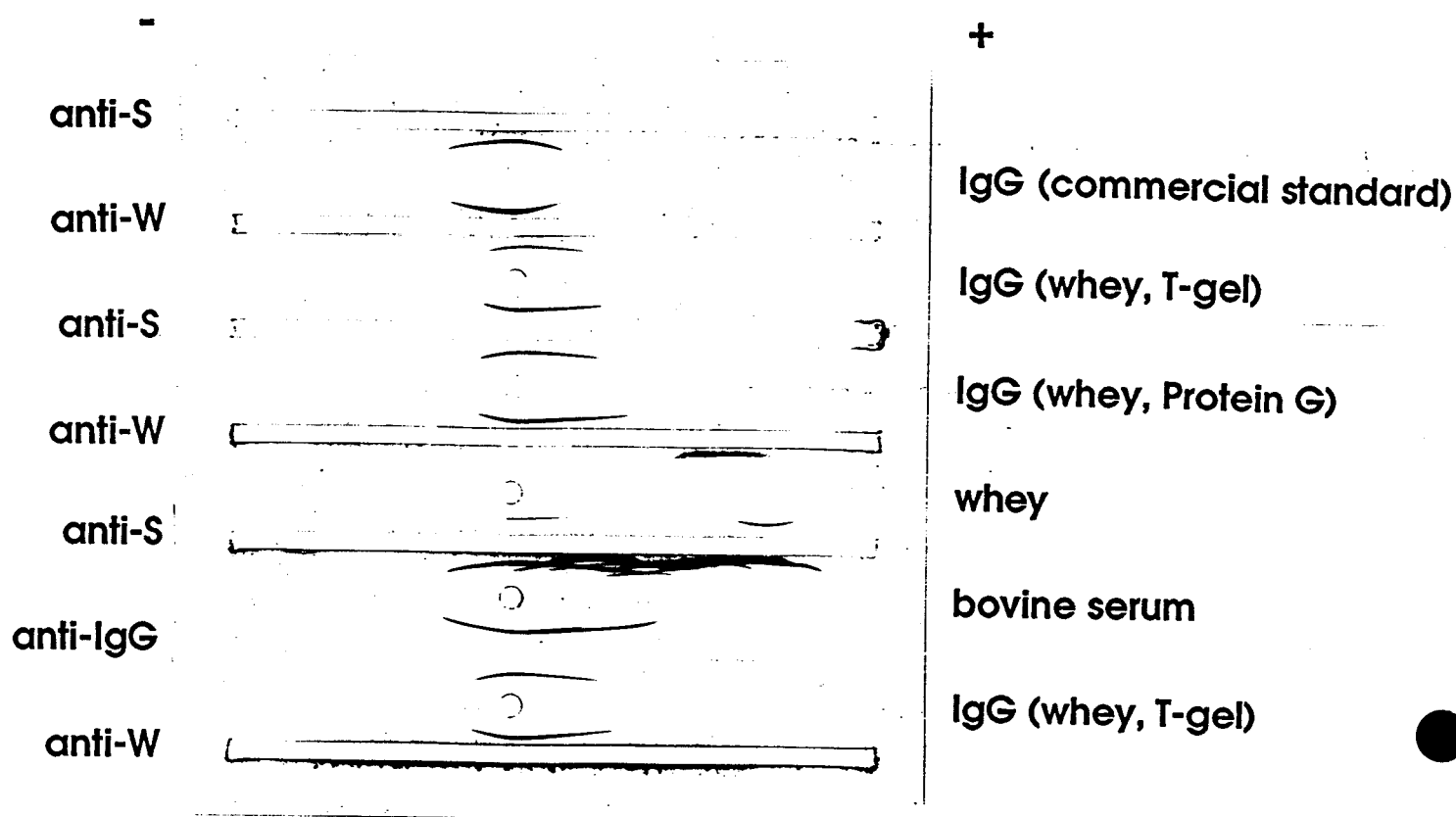


**Fig. 5:** Sodium dodecyl sulfate polyacrylamide electrophoresis (denaturing conditions) of fractions from chromatographic separation of sweet whey on T-gel and Protein G-Sepharose 4FF

SDS-PAGE was run on a 8 cm x 7 cm x 0.75 mm gel (stacking gel: 4 % acrylamide, separating gel: 12 % acrylamide), using the Laemmli buffer system at constant voltage 200 V.

1=  $\alpha$ -lactalbumin, 2=bovine IgG (standard; H= heavy chain, L= light chain), 3= bovine whey IgG from T-gel chromatography at 20 °C (see Fig. 4, fraction I), 4= bovine whey IgG from T-gel chromatography at 40 °C, 5= bovine whey IgG from Protein G-chromatography, 6= IgG-depleted whey after T-gel chromatography (see Fig. 4, fraction W), 7= IgG-depleted whey after Protein G-chromatography, 8= whey, 9= molecular weight standards, 10=  $\beta$ -lactoglobulin

## Immuno-electrophoresis of Bovine Immunoglobulin G purified from whey



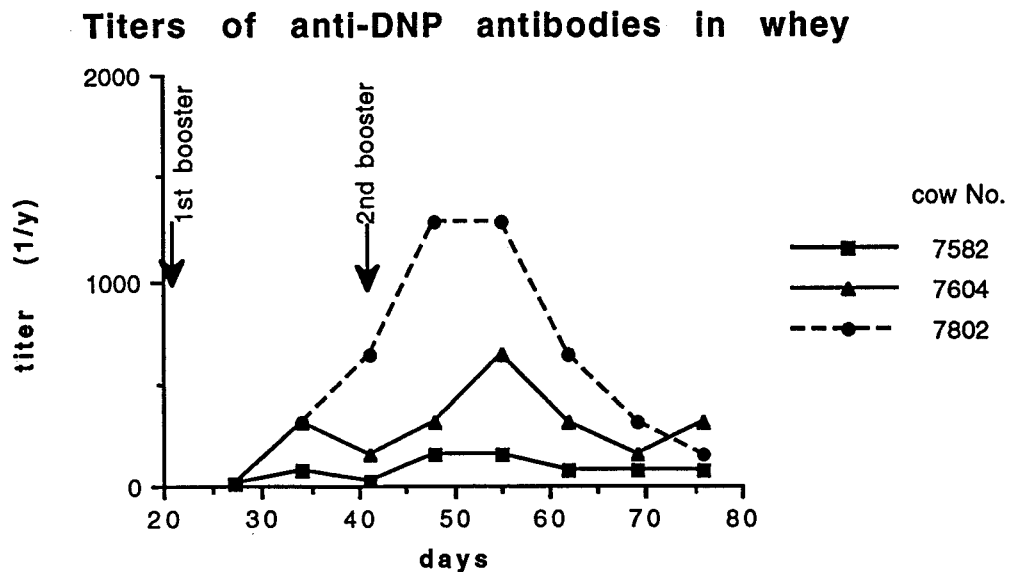
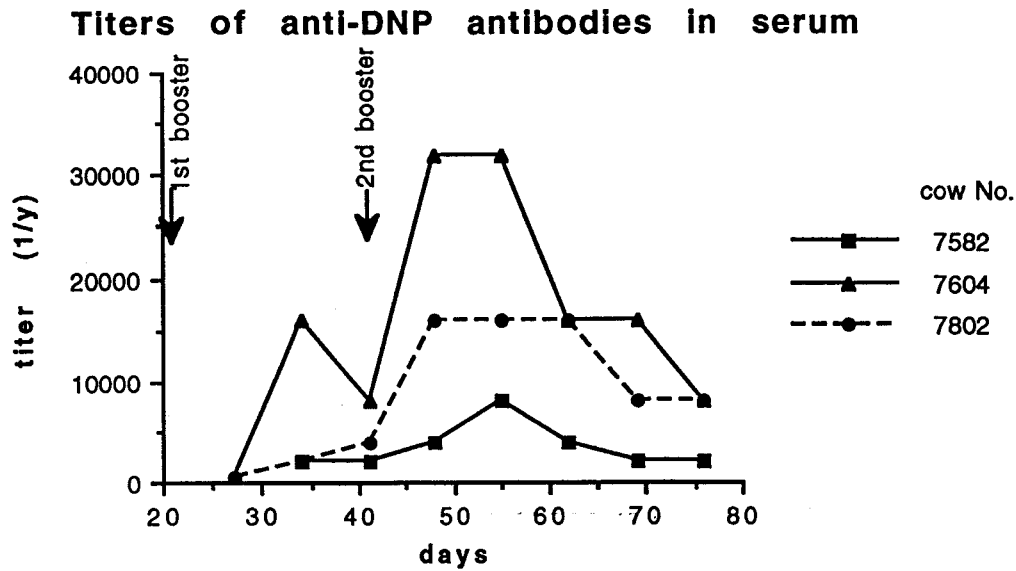
anti-S      anti-bovine whole serum, rabbit

anti-IgG    anti-bovine IgG, rabbit

anti-W      anti-whey proteins, rabbit

Fig. 6: Immuno-electrophoresis of IgG from whey

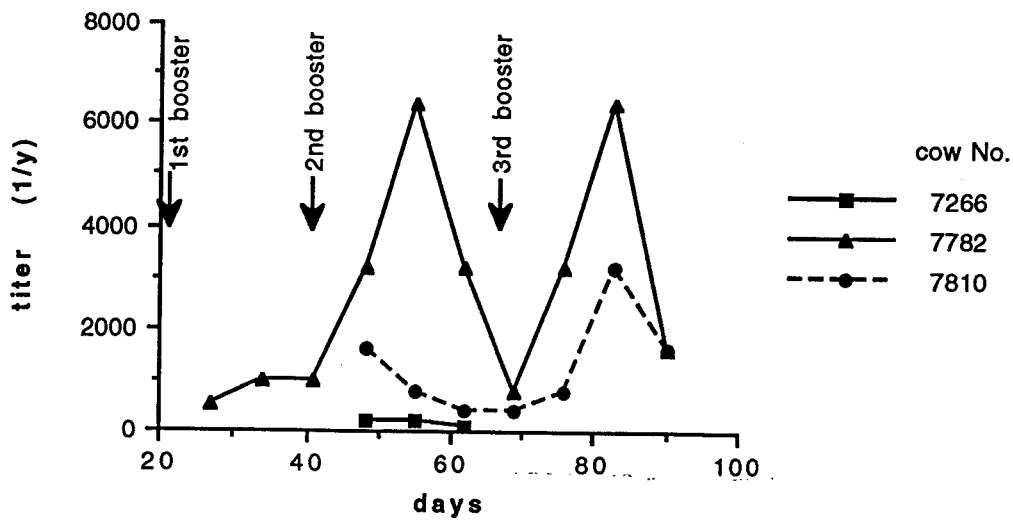
Immuno-electrophoresis was run on 10 cm x 10 cm x 0.1 cm agarose gel (2 % agarose solution in 0.06 M barbital HCl buffer, pH 8.6 / 0.002 % Thimerosal) at constant voltage 60 V (6 V/cm) for 3 hours. Immunodiffusion step (with antisera: anti-bovine whole serum, rabbit; anti-bovine IgG, rabbit; anti-whey proteins, rabbit) was performed at 4 °C for 24 hours, then the gel was dried and stained with Coomassie Brilliant Blue R-250.



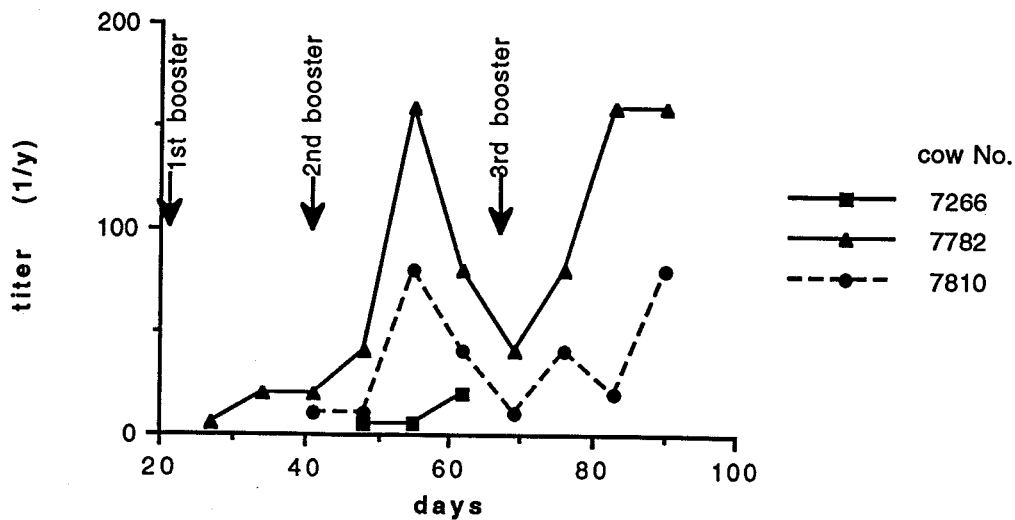
**Fig 7:** Antibody response in sera and whey from cows immunized with DNP-KLH

Cows were immunized with DNP-KLH in combination with Imject Alum (aluminum hydroxide) adjuvant, and boosted on days 21 and 42. Anti-DNP antibody was measured by ELISA. There was no antibody response in sera or whey from control cows (not shown).

### Titers of anti-mouse IgG antibodies in serum



### Titers of anti-mouse IgG antibodies in whey



**Fig 8:** Antibody response in sera and whey from cows immunized with mouse IgG

Cows were immunized with mouse IgG in combination with Imject Alum (aluminum hydroxide) adjuvant, and boosted on days 21, 42, and 66. Anti-DNP antibody was measured by ELISA. There was no antibody response in sera or whey from control cows (not shown).

**Project Title:** Milk Protein Interactions and Gelation During Thermal Processing

**Personnel:** Dr. Rodney J. Brown, Professor, Dept. of Nutrition and Food Sciences and Dean, College of Agriculture, Utah State University.

Dr. Donald J. McMahon, Associate Professor, Dept. of Nutrition and Food Sciences, Utah State University.

Dr. Mohan I. Reddy, Research Assistant Professor, Dept. of Nutrition and Food Sciences, Utah State University.

**Funding:** Western Center for Dairy Proteins Research and Technology

**Project Period:** July 1, 1993 through June 30, 1996

**Objectives:**

The main objectives of the project are (i) to determine the role of heat-induced complex between micellar  $\kappa$ -casein and  $\beta$ -lactoglobulin on the age gelation of UHT milk, and (ii) to study the heat-set gelation of non-micellar casein components in mixed protein-protein and protein-polysaccharide gels. The specific objectives of the project are to:

1. Develop a method to isolate the heat-induced complex between micellar  $\kappa$ -casein and  $\beta$ -lactoglobulin in milk.
2. Quantify the extent of complex formation between micellar  $\kappa$ -casein and  $\beta$ -lactoglobulin and its dissociation from casein micelles during the storage of concentrated UHT milk and correlate the extent of dissociation with gelation time.
3. Measure non-micellar casein-casein interactions that lead to their gelation as a function of temperature and solution composition.
4. Determine the nature of mixed casein-protein and casein-polysaccharide gelation.

**Results:**

**Objective 1: Development of a method to isolate the heat-induced complex between micellar  $\kappa$ -casein and  $\beta$ -lactoglobulin in milk.** It is well established that heat-induced interaction between micellar  $\kappa$ -casein and  $\beta$ -lactoglobulin occurs when milk is heated above 80°C. Heated skim milk was fractionated by ultracentrifugation at 100,000 x g, 25°C to separate casein micelles (along with micellar  $\kappa$ -casein/ $\beta$ -lactoglobulin complex) from whey proteins. Casein pellet thus obtained was dissolved in urea buffer, pH 6.8 and chromatographed on a gel filtration column using Sephacryl S-400. The peak fractions were pooled, concentrated and subjected to SDS-PAGE under reducing and non-reducing conditions. Based on the SDS-PAGE results, peak-I ( $V_e=25$  ml) which was absent in casein micelles fractionated from unheated milk, was identified as the heat-induced complex between  $\kappa$ -casein and  $\beta$ -lactoglobulin.



### **Impact of Research:**

Heat-induced interaction between micellar  $\kappa$ -casein and  $\beta$ -lactoglobulin is presumed to inhibit the age gelation of UHT milk. It is also suggested that the dissociation of the complex from casein micelles influences the age gelation of UHT milk. However, the dissociation of the complex during the storage of UHT milk has not been demonstrated and the exact mechanism of age gelation has not been worked out. This study is designed to elucidate the role of  $\kappa$ -casein/ $\beta$ -lactoglobulin complex on the age gelation of UHT milk, so that appropriate measures could be taken to prolong its shelf-life.

Formation of reversible gels with moderate heating (50-60°C) has been observed in calcium caseinate dispersions. This type of gel formation is rare among globular proteins and is only possible because of the unique structural features of caseins, namely, random coil structure and presence of ionic, hydrophobic, and ester phosphate groups in the polypeptide chain. These gels could have potential applications in the development of low fat foods. This study will provide new insights into the heat-set gelation of caseins as function of temperature and solution composition in mixed casein-protein and casein-polysaccharide gels and leads to the increased utilization of caseins in new food formulations.

### **Publications:**

Reddy, I. M., D. J. McMahon and R. J. Brown. 1994. A method for the isolation of heat-induced  $\kappa$ -casein/ $\beta$ -lactoglobulin complex from heated milk. (In Preparation)

**Project Title:** Improvement of lowfat Cheddar cheese through identification, isolation and analysis of enzymes and metabolites produced by adjunct cultures during accelerated Cheddar cheese flavor development.

**Personnel:** Jeffery R. Broadbent, Dept. Nutrition and Food Sciences, Utah State University.  
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**Funding:** Western Center for Dairy Protein Research and Technology  
Utah Agricultural Experiment Station

### Introduction

Current trends in the American diet clearly indicate that low-fat dairy products will be one of the most important research areas of the 1990's. Unfortunately, traditional cheese flavor is presently not available in reduced-fat varieties, and the inferior flavor and texture of these varieties limits their acceptability among consumers. In general, the lower the fat content, the more difficult it is to produce a cheese similar in quality to full-fat cheese. Starter cultures and media which perform well in the production of full-fat cheese often are not suited to low-fat varieties. As a result, there is a pressing need to develop specialized culture systems that overcome low-fat cheesemaking constraints. Development of new starter systems for low-fat products would be facilitated by more sophisticated knowledge of the role microbial enzymes and metabolites play in cheese flavor development. Studies of cheese maturation have revealed that nonstarter lactobacilli dominate curd microflora during the ripening period and thus are likely to contribute heavily to cheese flavor development. This hypothesis is supported by studies which demonstrated that certain strains of lactobacilli can be used as adjunct starter cultures to improve and accelerate cheese flavor development. Efforts to develop starter systems that improve flavor development in low-fat cheese would be greatly facilitated by a more sophisticated understanding of the role individual microbial enzymes and metabolites play in the maturation process. The most expedient way to approach such a complex problem may be to focus research efforts on cheese flavor adjunct cultures. These bacteria accelerate cheese flavor

development and are used industrially to obtain high quality reduced fat (33% fat reduction) cheese.

This proposal represents a unique collaborative effort between researchers at the Western Center for Milk Proteins Research and Technology at Utah State University, the Center for Dairy Research at the University of Wisconsin-Madison, and members of the Minnesota-South Dakota Dairy Foods Research Center at the University of Minnesota. Together, we seek to systematically identify and characterize key enzymes and metabolites which enable flavor adjunct cultures to accelerate and enhance flavor development in lowfat cheese. Elucidation of key biochemical pathways involved in both desirable and undesirable cheese flavor production would have immediate application in the development of starter systems for manufacture of high-quality low-fat Cheddar cheese.

**Objectives:**

1. To systematically characterize enzymes and metabolites implicated in cheese flavor development in starter and flavor adjunct bacteria.
2. To track the microbial conversion of milk proteins into peptides, amino acids and volatile flavor compounds in ripening cheese made with and without culture adjuncts to identify key flavor components which develop more rapidly.
3. To evaluate the role and specificity's of primary proteolysis on cheese flavor development.
4. To characterize the influence of peptidase activity from lactococci, *Lactobacillus casei*, and *Brevibacterium* spp. on cheese flavor development.
5. To characterize the enzymology of amino acid degradation in lactococci, *Lactobacillus casei*, *L. helveticus*, and *Brevibacterium* spp.
6. To characterize a-dicarbonyl production in flavor adjuncts.

**Results:**

Research to date has focused on objectives 1-4 as listed above. Forty-eight 550 lb vats of 50% reduced fat Cheddar cheese were manufactured at the University of Wisconsin using a single lactococcal starter or a starter-adjunct pair (Table 1). Starters selected for the study include two commercially used lowfat cultures and one strain known to produce bitter cheese. The adjuncts included four commercial *Lactobacillus* flavor adjuncts and two *brevibacteria*. In an effort to reduce extraneous influences on flavor development in the cheese, all vats were manufactured under conditions previously shown to minimize contamination by nonstarter lactic acid bacteria. Samples were collected for time 0 analyses, and the remainder was vacuum packaged and stored at 4-7°C for ripening. At monthly intervals, samples of each cheese were removed and analyzed for microbiological, enzymatic and chemical attributes (Table 2). To link changes in cheese composition with flavor properties, expert and consumer taste panels were performed after 2, 4 and 6 mo of ripening. Statistical results from consumer taste panels have

demonstrated that both starter and adjunct made significant contributions to flavor development. Efforts are now underway to draw correlations between taste panel conclusions and the chemical, enzymatic and microbiological data we have collected on each cheese over time.

TABLE 1. Starter-adjunct combinations used in the study<sup>1</sup>.

Starter	<i>Lactobacillus casei</i>		<i>L. helveticus</i>		<i>Brevibacterium linens</i>		
MM100	LC201	LC202	LH32	LH212	BL1	BL2	Control <sup>2</sup>
MM11	LC201	LC202	LH32	LH212	BL1	BL2	Control <sup>2</sup>
MM210	LC201	LC202	LH32	LH212	BL1	BL2	Control <sup>2</sup>

<sup>1</sup>All combinations were manufactured in duplicate.

<sup>2</sup>Duplicate starter-only control vats were prepared at the beginning and end of the make schedule.

TABLE 2. Characterization of cheese flavor attributes in 550 lb. vats of lowfat Cheddar cheese.

<u>USU</u>	<u>UW</u>	<u>UM</u>
Sensory analysis and statistics (consumer preference panels)	Sensory analysis and statistics (trained panels)	Free fatty acids (GC)
Free amino acid content (CE, amino acid analysis)	Non starter bacteria levels (microbiological)	Ammonia content (GC)
Peptide content (CE)	Starter levels (microbiological)	H <sub>2</sub> S content (GC)
<i>p</i> -cresol, indole and skatole (CE)	Adjunct levels (microbiological)	Methanethiol content (GC)
Aminopeptidase activity (colorimetric)	Redox potential (potentiometer)	Levels of other sulfur-containing volatiles (GC)
X-PDAP activity (colorimetric)	Methylglyoxal, glyoxal & diacetyl (HPLC)	
	D/L lactic acid (commercial kit)	

### Significance to the Dairy Industry

Future acceptance and demand for low-fat cheese will be heavily dependent on the availability of high-quality products. Solutions to the flavor and textural problems that have dogged low-fat cheese manufacture will require a more comprehensive understanding of the role other microbial enzymes and metabolites play in cheese flavor development. This proposal seeks

to identify and characterize specific enzymes and metabolites which enable cheese flavor adjunct cultures to accelerate cheese flavor development. Identification and characterization of these properties will facilitate the development of low-fat starter systems, through strain combinations or recombinant DNA technology, for the manufacture of high-quality low-fat cheese. Low-fat cheese with organoleptic qualities of full-fat varieties will increase consumer acceptance of low-fat dairy products and expand consumer demand for these goods to individuals that avoid cheese for reasons of diet and the absence of high quality low-fat alternatives.

**Publications**

none in 1993-94

**Abstracts**

Strickland, M. and B.C. Weimer. 1994. Use of capillary electrophoresis for detection of phenylalanine, tyrosine, tryptophan, indole, skatole, p-cresol, caseins and peptides in Cheddar cheese. *J. Dairy Sci.*(Suppl. 1) 77:

**Project Title:** Selection of lactococcal starter strains for use in low fat cheese manufacture based on their hydrolysis of milk proteins.

**Personnel:** Bart Weimer, Dept. of Nutrition and Food Sciences, Utah State University  
Bart DeVries, Dept. of Nutrition and Food Sciences, Utah State University

### Introduction

Future acceptance and increasing demand for low fat cheese will be heavily dependent on the availability of starter cultures that consistently produce a high-quality, flavorful product. Development of these cultures will in turn be dependent upon identification of key metabolic functions, which promote desirable cheese flavor, texture, and body, and their relationship to bacteriophage (phage) resistance. Production of full fat cheese is still plagued with problems associated with bacteriophage during cheese making and low fat cheese makers will be faced with fewer starters to choose from that will make an acceptable product. This dichotomy between phage resistance and starters with desirable production characteristics has left the cheese maker with a hard full of strains for manufacture of low fat cheese.

Low fat cheese manufacture presents unique demands for lactococcal starter cultures in terms of flavor production and phage resistance. Traditional strains, commercially available, have proven to produce inferior low fat cheese even though they produce high quality full fat cheese. One important difference for low fat cheese starters is that the strains used must produce acid at a slow rate, so acid development can be controlled during cheese making.

Derivation, widely used in New Zealand and Australia to obtain phage resistant variants, yields many slow acid-producing mutants that have been discarded in the past. However, phage derivation also changes many biochemical characteristics that influence proteolysis and subsequent flavor development. These changes also alter optimum growth parameters for bulk starter production when used for cheese making. To consistently produce a high quality, low fat cheese commercially, it is necessary to isolate and characterize starter cultures specifically for use in low fat cheese. It is important to develop a bank of cultures suitable for use by commercial cheese manufacturers that will be phage resistant.

In Australia, derivation has been used successfully in factories to produce many phage resistant starters used industrially for many years. This technique has provided a continuing supply of uncharacterized starters that have varying genetic and metabolic characteristics as compared to the parent. A need exists to have a bank of phage resistant starters for low fat cheese production that are characterized with regard to proteinase, peptidase, lipase, and other key enzymes that contribute to cheese flavor, texture and body of cheese.

### Objectives

- A. Select slow acid-producing starter strains (both *Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris*) that have proven quality records in full fat cheese manufacture. Isolates will be compared to benchmark strains that are currently used for low fat cheese production
- B. Examine effect of media and environmental parameters on growth, proteinase, and peptidase activity.

- C. Characterize effects of derivation for phage resistance on the phenotypic expression of  $\beta$ -galactosidase, proteinase, peptidase, and metabolism of parents and phage-resistant derivatives.

## Results

Slow acid-producing phage resistant starter strains have been isolated from three strains of lactococci used to make good quality low-fat and high fat Cheddar cheese. These phage-resistant strains have been partially characterized for phenotypic changes due to selection (Table 1). Changes are mutant dependent and are found in enzyme systems associated with cheese flavor which influence the commercial utility for flavor development and acid production in cheese production. Characterization of some traits are still underway for the mutants. Initial results indicate  $\beta$ -galactosidase, proteinase, peptidase, and metabolism of the mutants are significantly different from the parent strain.

Early results from objective 2, determination of the influence of media composition, indicate media has a significant effect on the peptidolytic and lipolytic activity of the strains tested. This is an important finding and suggests bulk media composition will impact starter culture performance and flavor development via enzyme system activation during bulk starter growth. These data provide insight into which enzymes can be manipulated with media composition and what impact they will have on cheese flavor when bulk starter cultures are used in cheese manufacture.

Table 1. Characteristics that will be determined for parents and derivatives.

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Acid production (colometric assay and pH [Pearce, 1969])
General metabolic characteristics - 95 biochemical attributes (Biolog)
Salt tolerance - 0 to 5% (colometric assay)
Arginine metabolism (Differential broth)
Phage host range (colometric assay, plate assay)
General proteinase activity (chromogenic assay, capillary electrophoresis)
Total aminopeptidase activity (chromogenic assay)
Lipase/esterase activity (chromogenic assay)
Genetic characterization (Pulse field Gel Electrophoresis, plasmid profile)
$\alpha$ -dicarbonyl (King Test [Vasavada and White], HPLC, capillary electrophoresis)
<i>p</i> -cresol, indole, and skatole (HPLC, capillary electrophoresis)
Glutathione - cellular content (HPLC [Fernandez and Steele, 1993])

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## Significance to the Dairy Industry

Phenotypic changes due to selection of phage resistant mutants significantly impact the flavor of cheese. This work highlights the importance of the culture characterization and

suggests metabolic traits that influence cheese flavor can be controlled and may need to be accounted for when cultures are rotated for phage resistance. Determination of the growth conditions which influence the enzyme systems that contribute to desirable cheese flavor will lead to recommendations to the cheese maker that will minimize undesirable flavor systems while maximizing desirable characteristics.

**Publications**

none in 1993-1994

**Abstracts**

none in 1993-1994



**Time and processing effects on the water interaction with the protein-reduced fat matrix**

Report to the western dairy foods research center

Antonio Torres and Conly L Hansen, Co-Principal Investigators

**INTRODUCTION**

Manufacture of high quality low-fat cheese (with  $\geq 50\%$  reduction in fat compared to its full-fat counterpart) has proven difficult because these types of products typically lack characteristic flavor and texture attributes of full fat cheeses.

When a significant percentage of fat is removed from milk for the manufacture of low-fat cheeses, the rheological properties and texture of the cheeses are adversely affected. A common defect is in the cheese texture. Removal of fat from milk increases the protein content. It is thought this makes the texture of cheese too hard and plays a role in the lack of flavor development in low-fat cheeses. There are many factors that affect the quality of low-fat cheese. One factor that must be explored is to determine how manufacturing conditions affect microbial growth and protein functionality and breakdown in low-fat cheeses. The principal investigators listed above are part of the team researching how to improve the quality of low-fat cheese.

## Research Objective 3

Determine how manufacturing conditions affect microbial growth and protein functionality and breakdown in low fat cheeses.

### Research Strategy 3.1

Fat reduction effect on the water interaction with the protein-fat matrix.

#### Specific Objectives:

- A. Prepare cheese at minimum, 75% reduced, 50% reduced, 33% reduced and full fat level following curd handling procedures used in industry. (Year 1 and 2).
- B. Determine during early storage (under 30 days) and at constant temperature the changes of the state of water in the reduced fat - protein matrix. Measurements used include differential scanning calorimetry (DSC) and moisture. (Year 1 and 2).
- C. Determine during early storage (under 30 days) the state of water in cheese slabs as affected by temperature gradient and location in the slab. (Year 3)
- D. Determine during early storage (under 30 days) structural changes with time and temperature of the cheese matrix by electron microscopy. (Year 1 and 2)
- E. Develop and validate a moisture transfer model by determining the driving forces affecting free whey generation and its movement in the cheese matrix and quantifying the appropriate model parameters. (Year 2 and 3).
- F. Make process recommendations for the handling of low fat cheese curd and pressing into blocks. (Year 3).

#### KEY DELIVERABLES (first year):

- 1. Cheese microstructure changes in a few days from a porous sponge-like material with interconnected channels (diameter  $\approx 10 \mu\text{m}$ ) in freshly pressed cheese to a continuous protein matrix with embedded fat particles. This provides a new interpretation for the locking of moisture variability in Cheddar cheese blocks and should lead to processing conditions reducing this variability.
- 2. The presence of interconnected channels during the time that moisture gradients are established in a cheese block suggest that moisture movement within cheese blocks may be described as whey flow in a porous media.
- 3. Temperature differences affect rate of free whey production but do not explain its movement within a cheese block. This suggests that moisture gradients could be reduced even if temperature gradients are not eliminated.

WORK DONE: Experimental work completed (first year):

1. Cheese has been prepared at three different fat levels:
  - minimum fat
  - 50% reduced
  - full fat cheese
  
2. Curd treatments included so far have been:
  - A - stirred curd
  - B - curd and whey mixture cooled to 70° F (\*)
  - C - curd and whey mixture cooled to 80° F (\*)
  - D - curd washed with cold water to 70° F
  - E - curd washed with cold water to 80° F
  - F - curd and whey mixture cooled to 70° F (\*) and minimum water rinse
  - G - curd and whey mixture cooled to 80° F (\*) and minimum water rinse
  - H - stirred curd (vat A replicate for additional variability assessment)(\* ) chilled water through the jacketed wall
  
3. Sample handling procedures were as follows. Each vat yielded one cheese block (≈ 20 lb) which was cut into small size chunks (2"x1.5"x5") for faster equilibration to storage temperature (under 2 hours).
  - Storage temperature: 4° C, 13° C, and 25° C.
  - Storage time: 1, 2, 5, 10 and 21 days.
  - Measurements included DSC analysis, moisture content and pH.

## EXPERIMENTAL RESULTS

The data obtained and analyzed so far indicates that the volume of whey expelled from cheese is affected by temperature and curd handling procedures. It is important to note that such expulsion occurs even in the absence of temperature differences within the cheese samples. For example, Figure 1 (minimum fat cheese, i.e., ≈ 1% fat) shows a large drop in moisture content in samples stored at 25° C even though the initial sample temperature is approximately the same as the storage temperature. Furthermore, the small size chunks (2"x1.5"x5") used in our experiments can reach 4° C and 13° C in ≈ 2 hours. Therefore, whey movement to the block surface occurs even in the absence of temperature gradients. The driving force for this whey movement will be determined in years 2 and 3 of this project.

A related experiment was the preparation of thin curd layers (Figure 2) stored at constant temperature for approximately 24 hours. These thin layers were pressed at each sampling time and the amount of whey expelled was estimated by recording changes in the weight of the pressed curd. The curves obtained suggest a first order kinetics behavior in the 5-30° C temperature range. A comparison between Figures 1 and 2 shows that in the small size chunk experiments no significant differences are observed between 4° C and 13° C. This observation might reflect the relatively large distance that the whey needs to travel to reach the surface in the case of the cheese chunks as compared to thin curd layers. Also, in the latter experiment the samples were pressed at each sampling time. This preliminary experiment suggests a mean to differentiate between free

whey generation and free whey movement. Combining this information with pH changes (Figure 3) we will propose a mechanistic model for moisture variability in natural cheese blocks.

A mechanistic interpretation of moisture movement in natural cheese requires information on the moisture matrix interaction. Differential scanning calorimetry (DSC) can provide such information. A DSC unit donated by the Technology Center at Kraft General Foods to support this project is being used to quantify the energy involved in phase transitions as a function of storage time and temperature, fat level, and curd handling (Figures 4 and 5). The ice melting peak area remains more or less constant after an initial drop (Figure 4). Note that peak area, expressed as energy (J) per sample size (gram of cheese) depends on the moisture content of the sample. Therefore, a better parameter is the amount of energy involved per gram of water divided by the amount of energy per gram of pure water. This parameter ("free water index", FWI) reflects the water fraction participating in the phase transition. Figure 5 shows changes in FWI occurring early during storage time. We are currently determining whether FWI is affected by curd handling technologies.

Electron microscope (SEM) observations show cheese microstructure changing in a few days from a porous sponge-like material with interconnected channels (diameter  $\approx 10 \mu\text{m}$ ) in freshly pressed cheese to a continuous matrix with embedded fat particles. This provides a new interpretation for the locking of moisture variability in Cheddar cheese blocks. Whey movement is reduced to near zero when the interconnected channels disappear.

In summary, the first phase of this study shows that factors involved in moisture migration in Cheddar cheese block are active during storage (probably less than 5 days). Moisture movement in chunks, syneresis in thin curd layer, DSC determinations, and SEM structure changes were observed only during early sample storage.

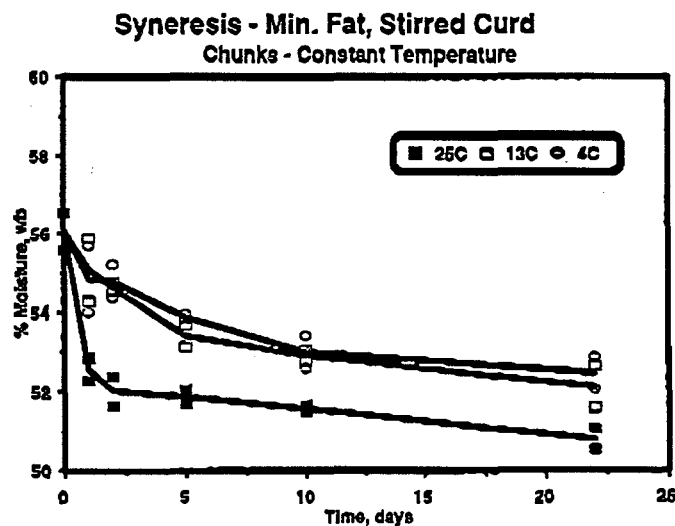


Figure 1

**Syneresis - 33% Reduced, Stirred Curd**  
Thin Layer - Constant Temperature

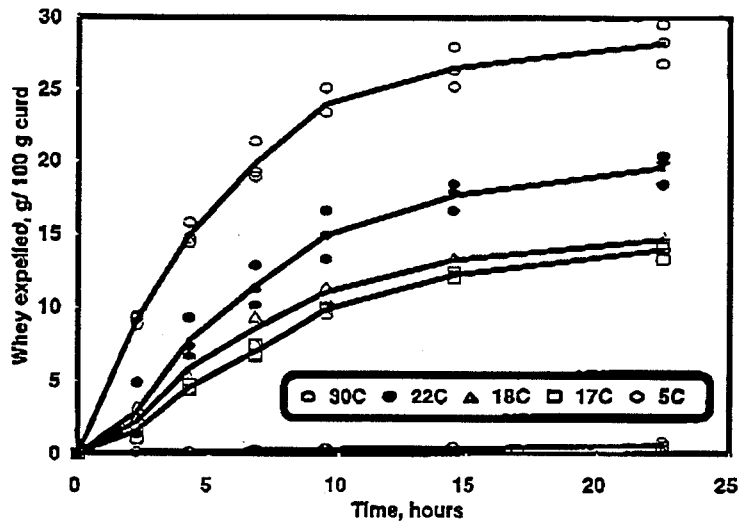


Figure 2

**pH - Min. Fat, Stirred Curd**  
Chunks - Constant Temperature

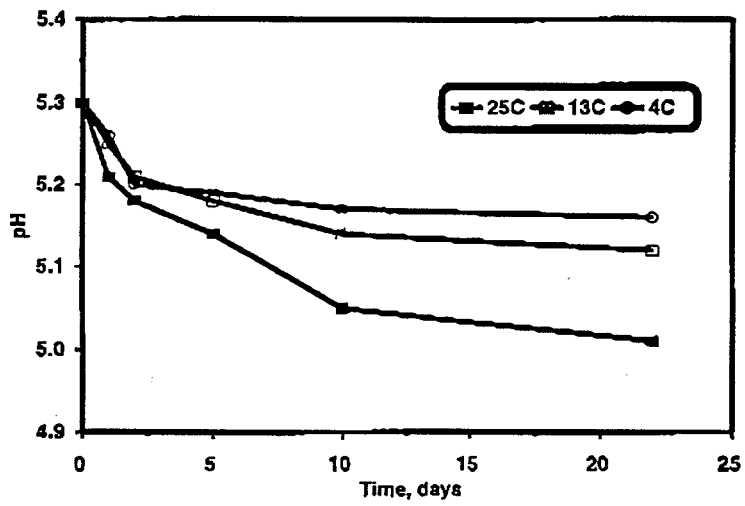


Figure 3

**DSC peak - Min. Fat, Stirred Curd**  
**Chunks - Constant Temperature**

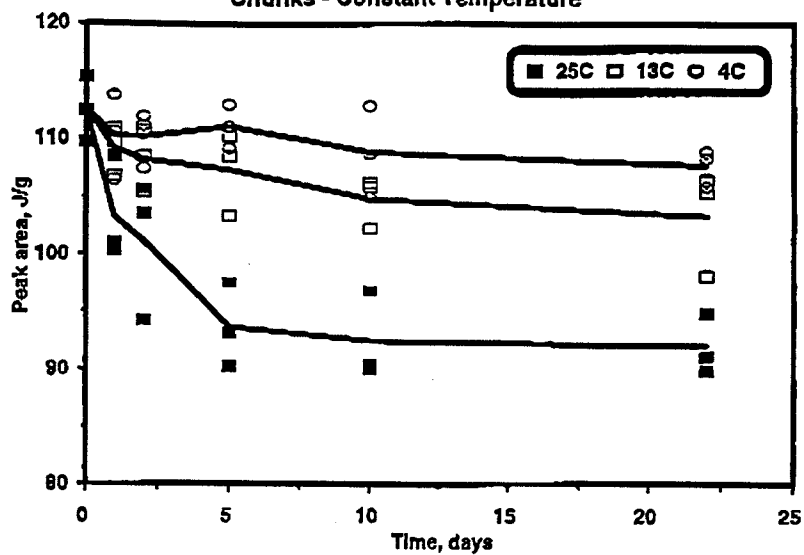


Figure 4

**FWI - Min. Fat, Stirred Curd**  
**Chunks - Constant Temperature**

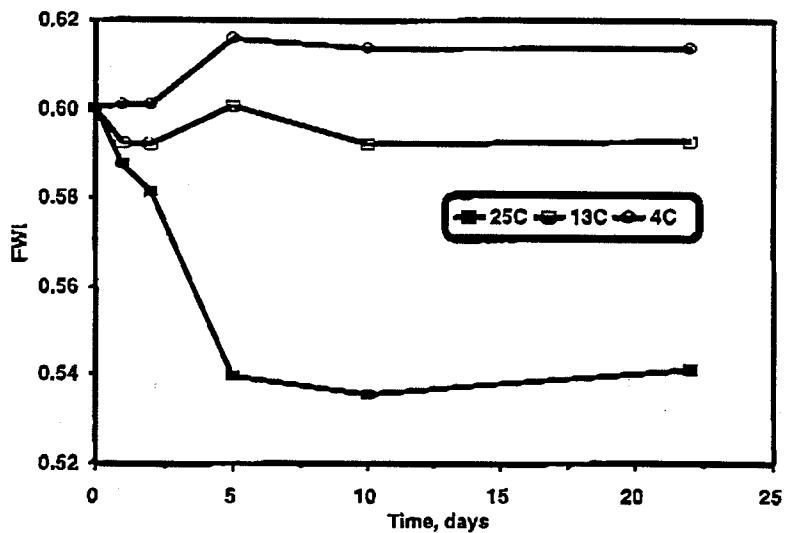


Figure 5

**Title:** Proteinase activities from new strains of *Lactococcus lactis* subsp. *cremoris*

**Personnel:** Bruce Geller  
Department of Microbiology  
Oregon State University

**Funding:** Western Center for Dairy Protein Research and Technology

#### Introduction

The objective of the project is to characterize and evaluate recently isolated, unique strains of *Lactococcus lactis* subsp. *cremoris* for proteolytic specificities, and for their potential use in low fat cheeses. The characterization focuses on proteolytic activity because it is necessary for the acid production required in milk fermentations, and is a determinant of the flavor in cheeses. Ultimately, our goals are to identify the characteristics among these new strains of *L. lactis* subsp. *cremoris* that are favorable for making low fat cheeses, and to understand the chemical and biological differences between strains that account for the differences in their abilities to make high quality cheeses.

Nearly all commercial and laboratory strains of *L. lactis* subsp. *cremoris* are derivatives of just a few unique strains. Many attempts to isolate different strains have failed, leaving the fermented dairy foods industry with a limited variety of genetic and biochemical characteristics in this subspecies.

Recently, two labs in our department have collaborated on a NDPRB-funded project, and isolated 28 new strains of *L. lactis* subsp. *cremoris*. Five of these strains have been tested by seven independent methods, and are genetically and biochemically different from one another, as well as from any of the known *cremoris* strains. Our lab is characterizing these strains in the following way: 1) determining the specificities of the proteinases by the electrophoretic pattern of the products of casein hydrolysis, 2) making and testing a low fat cheddar cheese and 3) cloning and sequencing the gene(s) for proteinases with unique proteolytic specificities.

#### Specific Objectives

In broad terms, the objective of the project is to characterize and evaluate 28 recently isolated and unique strains of *Lactococcus lactis* subsp. *cremoris* for proteolytic activities, and for potential use in low fat cheeses.

To accomplish this, the project has the following specific objectives:

- a) Determine the specificities of the proteinases by analyzing the cleavage products of the casein substrate.
- b) Make and analyze a low fat cheddar cheese using one or more of the new strains.
- c) Clone and sequence the gene(s) for proteinases with unique proteolytic specificities and favorable cheese-making characteristics.

#### Significance

Our project is to characterize newly isolated strains of *Lactococcus lactis*

subsp. *cremoris*, starting with the one of the most important characteristics of the starter culture, the proteinase activity. There are many significant reasons why the proposed research will benefit the dairy industry. First, low fat cheese is now made from only a few acceptable strains of subsp. *cremoris*, and the proposed project would identify those newly isolated, unique *cremoris* strains with the proteinase characteristics required for commercial use. Second, our approach to the problem of a lack of commercially usable *cremoris* strains takes advantage of the natural pool of *cremoris* traits that have gone untapped. Much emphasis has been placed recently on modifying existing *cremoris* strains using genetic engineering, particularly by the European dairy research groups. However, our proposal would avoid the legal and commercial uncertainty of using genetic engineering, and characterize new, potentially usable, and naturally-isolated strains of subsp. *cremoris*. Third, preliminary tests have shown that the new strains have characteristics which are different from existing strains of *cremoris*, and which are favorable for cheese-making (such as fast acid production and phage-resistance). This indicates that the new strains are potentially usable for making cheese, and have characteristics previously unknown in other *cremoris* strains. Fourth, our approach is to understand a chemical/enzymatic basis for differences between the strains' abilities to make a better cheese. This would give the industry a rational way of screening new strains in the laboratory, without having to test each strain by trial and error in a cheese vat. Fifth, by isolating the proteinase genes from these "new" strains, they could be transferred to other strains to produce unique combinations of characteristics important for cheese-making.

### Results

Twenty-eight of the new *cremoris* isolates have been screened for their proteolytic degradation patterns of casein. Two appear to have unique patterns that are different than any of the three known patterns. Thus, at least two of the new strains appear to be different in their proteolytic degradation of casein than any of the existing *cremoris* strains. We are currently screening more of the strains for their casein degradation patterns, including two that taste-tested bitter. We have also started a more detailed characterization of the proteinases with unique patterns, which includes a kinetic analysis of the crude proteinase preparations, and an analysis of the degradation of the individual casein subunits. In collaboration with the Sandine and Giovannoni labs and Tillamook Dairy, we are planning to make and test a low fat cheddar cheese using at least one of the strains that we have identified as having unique proteolytic activity.

### Deliverables

For FY95 (07\01\94-06\30\95), the deliverables are:

1. Identify strains of *Lactococcus lactis* subsp. *cremoris* with unique patterns of casein degradation.
2. Make a low fat cheese with at least one of the new *cremoris* strains, and analyze it by objective and subjective criteria.
3. Identify and characterize the peptides formed from the hydrolysis of casein by the unique proteinases of the new *cremoris* strains.



## PROGRESS REPORT

**Project Title:** Preliminary Examination of Hazelnut Enzyme Extracts for Flavor Enhancement of Reduced Fat Cheddar Cheese

**Personnel:** Floyd W. Bodyfelt  
Michael H. Penner  
Dan Smith  
Dept. of Food Science and Technology, Oregon State University

**Funding:** Western Center for Dairy Proteins Research and Technology

### Introduction:

In previous work at the OSU Department of Food Science and Technology (~1950), a crude enzyme extract of hazelnut enzymes (HEE) was applied to Cheddar cheese curd for the purpose of developing aged cheese flavor within 1-2 weeks time. This study was terminated primarily because the protease and/or lipase activity could not be controlled; developed flavors became too intense. Recent application of high hydrostatic pressure (2000-4000 atmospheres) treatment has demonstrated the potential for inactivation or control of enzyme systems without destroying sensory properties of cheese. Thus, this study is a preliminary examination of the feasibility of developing more appealing flavor and textural properties of HEE treated lowfat Cheddar cheese.

### Objectives:

1. Assay the protease and lipase activity and ascertain the extent of potential bitterness developed in 2% UHT milk with added hazelnut enzyme extracts.
2. Determine the optimum high pressure treatment for HEE inactivation.
3. Determine and describe the effects of high pressure treatment on the sensory and rheological properties of Cheddar cheese.
4. Characterize the proteolytic, lipolytic and flavor imparting properties of various hazelnut preparations.
5. Evaluate the suitability of propylene oxide sterilized hazelnuts as a source of HEE and "whole nut" preparation(s).
6. Study and refine methodologies for preparation and incorporation of potential flavor imparting hazelnut preparations onto cheese curd.
7. Conduct necessary cheesemaking trials to optimize incorporation of HEE and whole hazelnut preparation; ascertain proteolysis, lipolysis and sensory properties modification of lowfat cheese.

### Results:

Hazelnut meats were obtained from an Oregon processor (Westnut, Inc., Dundee). Hazelnut enzyme extracts (HEE) were prepared using (1) 1.0 M NaCl and (2) 0.05 M phosphate buffer (pH 7.0) with subsequent purification by salting out with ammonium sulfate. The aqueous portion of the extracts was obtained by centrifugation and filter sterilized (0.2  $\mu$ m). Cartons of 2% UHT milk (236 ml) were aseptically inoculated in duplicate with aliquots of each extract (4% by volume), incubated for 3 and 6 days at 7.2°C, and then examined for the appearance of proteolytic products using the OPA method of Church, *et al.* Both extracts produced significant amounts of proteolysis in the milk within three days. The detectable proteolytic products in milk treated with the NaCl extract were 3-4 times greater than the assay's background detection of amino acids and

peptides in untreated milk. Detectable proteolytic products in the phosphate buffer extract treated milk were 2-3 times above background. The phosphate buffer extract showed much better stability, as the NaCl extract lost almost half of its proteolytic activity when held at 4°C for 24 hours prior to inoculating the milk. Beyond day 3 of incubation the proteolytic activity in both extracts diminished markedly. Treatment of inoculated milk samples with high pressure (3500 atm for 30 min) terminated proteolytic activity.

A trained sensory panel did not detect bitterness or other off-flavors in sterile filtered HEE treated milk samples. Total plate counts showed them to be free of microbial contamination.

Lipase activity was estimated by titration of free fatty acids produced in HEE inoculated 2% UHT milk. Negligible amounts of fatty acids were detected, perhaps owing to the low milk fat. The NaCl hazelnut extract exhibited esterase activity toward artificial substrates of chain lengths C4 to C16. The phosphate extract is still to be tested for lipase/esterase activity.

Low fat cheddar curd was treated with 2% (volume of NaCl extract/weight of curd) HEE. To date, at ten weeks aging at 7.2°C, the sensory panel has not observed any difference in flavor characteristics between the HEE treated and control low fat cheddar. There is no significant difference in proteolytic products as measured by the OPA assay.

Microbial contamination of the hazelnuts and instability of the proteolytic activity in the HEE are two challenges that have been partially overcome. The microflora present on the hazelnut surface confounded the initial observations in milk. Hazelnuts treated with propylene oxide gas, provided by Westnut, have a much lower plate count while retaining most of the proteolytic activity. The use of nisin or irradiation of the HEE are also under consideration as controls of incidental contamination. Ideally, we hope to provide an extraction protocol that avoids the need for a somewhat cumbersome sterilizing filtration. As noted above, HEE is more stable in phosphate buffer than the NaCl solution. We are working to optimize both initial activity and stability of the extract. Freeze drying has also shown promise for the retention of enzyme activity in this extract.

#### **Significance to the Industry:**

Recent consumer trends toward consumption of lower fat content foods prompts the dairy industry to closely examine all technological approaches toward the development and production of higher quality, more appealing lowfat Cheddar cheese. This study represents another possible means of attaining this industry goal.

#### **Deliverables FY 95:**

It is our intent to determine an optimum method(s) to separate and utilize either hazelnut enzyme extracts and/or incorporate a whole hazelnut preparation (i.e. semi-flowable powder) for the potential treatment of lowfat Cheddar cheese curd for enhancement of lowfat cheese flavor properties.

**Project Title:** Development of Thermophilic Cultures for Manufacture of low-fat and non-fat Mozzarella Cheese.

**Personnel:** Donald J. McMahon  
Craig J. Oberg

**Funding:** WESTERN CENTER FOR DAIRY PROTEIN  
RESEARCH AND TECHNOLOGY  
Utah Agricultural Experiment Station

**Objectives:**

In the manufacture of low fat and non fat Mozzarella cheeses it has been well recognized that the moisture content of the cheese must be increased without having "weeping" of whey from the cheese after packaging. It has also been shown that low fat Mozzarella cheese takes a longer time to "mellow" and develop good melting properties. This process could perhaps be accelerated by increasing the proteolysis occurring in the cheese during storage. Both of these factors (increasing moisture and increasing proteolysis) can be manipulated through use of appropriate starter cultures.

- A. Develop thermophilic starter cultures, including *Streptococcus thermophilus*, *Lactobacillus helveticus*, *Lactobacillus delbruekii* ssp. *bulgaricus*, and *Lactobacillus casei* strains that will be suitable for use in the manufacture of low fat and non fat Mozzarella cheese.
- B. Manufacture low fat and non fat Mozzarella cheese using cultures developed in objective A that have the desirable physical properties of part-skim Mozzarella cheese.

**Results:**

Some cultures that have been reported to increase moisture content of Mozzarella cheese have been obtained. Also some Lac<sup>-</sup> Prrt<sup>+</sup> cultures have been obtained. The graduate students who are to work on this project did not commence their program until June 1994. Preliminary cheesemaking trials have been conducted but no results are available at this time.

**Publications:**

Nil

# WESTERN CENTER FOR DAIRY PROTEIN RESEARCH AND TECHNOLOGY

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Researching the Western  
U.S. Dairy Industry's Future

ANNUAL MEETING  
Sun Valley, Idaho  
July 28 - 29, 1994

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PI: Conly Hansen
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- ✓ Purification of monospecific polyclonal antibodies from bovine cheese whey.  
PI: Rodney Brown

Project Annual Reports (continued):

- ✓ Milk protein interactions and gelation during thermal processing.  
PI: Rodney Brown
- ✓ Improvement of low-fat cheese through identification isolation and analysis of enzymes and metabolites produced by adjunct cultures during accelerated cheese flavor development (low-fat cheese project).  
PI: Jeff Broadbent
- ✓ Selection of lactococcal starter strains for use in low-fat cheese manufactured based on their hydrolysis of milk proteins (low-fat cheese project).  
PI: Bart Weimer
- ✓ Time and processing effects on water interaction with the protein-reduced fat matrix (low-fat cheese project).  
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- Time and processing effects on water interaction with the protein-reduced fat matrix (low-fat cheese project)  
PI: Antonio Torres
- ✓ Proteinase activities from new strains of *Lactococcus lactis* subsp. *cremoris* (low-fat cheese project).  
PI: Bruce Geller
- ✓ Preliminary examination of hazelnut enzymes extracts for flavor enhancement of reduced fat Cheddar cheese (low-fat cheese project).  
PI: Floyd Bodyfelt
- ✓ Development of thermophilic cultures for manufacture of low-fat and non-fat Mozzarella cheese (low-fat cheese project).  
PI: Donald McMahon

Operational Advisory Committee business meeting agenda

**WESTERN CENTER  
ANNUAL MEETING - 1994  
JULY 28 & 29, 1994  
SUN VALLEY, IDAHO**

**AGENDA**

**July 28, 1994**

- 1:00 p.m. **INTRODUCTION**  
Western Center mission statement - Donald McMahon  
Direction of Center - Don McMahon  
Center review by NDB - NDB Staff  
Review of FY95 annual plan - Carl Brothersen  
Review of center operations - Carl Brothersen
- 2:00 p.m. **PROJECT REPORTS**  
**Mark Daeschel** - Influence of preadsorbed protein on adhesion of *Listeria monocytogenes* to dairy food contact surfaces.
- 2:15 p.m. **Gerald Schelling** - Using a natural process to improve milk quality and extend milk shelf-life through the reduction of lipid oxidation and off-flavor with tocopherol (vitamin E) supplementation to dairy cows.
- 2:30 p.m. **Donald McMahon** - Function of whey protein and lactose in age gelation of UHT-processed milk concentrate - Part 2.
- 2:45 p.m. **Donald McMahon** - Effects of iron fortification on chemical, physical, microbiological and nutritional properties of yogurt.
- 3:00 p.m. **Bart Weimer** - Interactions between milk proteins, starter cultures and hydrocolloidal milk fat replacers.
- 3:15 p.m. **Bart Weimer** - Production of extracellular proteases of *Brevibacterium linens* for use in low-fat cheese.
- 3:30 p.m. **BREAK**
- 4:00 p.m. **SEPARATE INTO COMMITTEES**  
Structure of Center - Chairman: Donald McMahon  
Research directions - Chairman: Floyd Bodyfelt  
Industry support - Chairman: Carl Brothersen  
Role of the Center in the West - Chairman: Mark Daeschel
- 7:00 p.m. **GROUP DINNER**

**July 29, 1994**

- 7:00 a.m. Continental breakfast
- 8:00 a.m. **PROJECT REPORTS**  
**Bruce Geller** - Bacteriophage-resistance gene replacement in *Lactococcus lactis*.

July 29, 1994, continued

- 8:15 a.m. **Yehia El-Samragy** - Development of high protein low-fat fermented foods from yogurt cheese.
- 8:30 a.m. **Conly Hansen** - Extrusion processing of milk proteins.
- 8:45 a.m. **Donald McMahon** - Rheology and microstructure of Mozzarella cheese.
- 9:00 a.m. **Rodney Brown** - Purification of monospecific polyclonal antibodies from bovine cheese whey.
- 9:15 a.m. **Rodney Brown** - Milk protein interactions and gelation during thermal processing.
- 9:30 a.m. **Jeff Broadbent** - Improvement of low-fat cheese through identification isolation and analysis of enzymes and metabolites produced by adjunct cultures during accelerated cheese flavor development. (low-fat cheese project).
- 9:45 a.m. **Bart Weimer** - Selection of lactococcal starter strains for use in low-fat cheese manufactured based on their hydrolysis of milk proteins (low-fat cheese project)
- 10:00 a.m. BREAK
- 10:30 a.m. **Conly Hansen** - Time and processing effects on water interaction with the protein-reduced fat matrix (low-fat cheese project).
- 10:45 a.m. **Antonio Torres** - Time and processing effects on water interaction with the protein-reduced fat matrix (low-fat cheese project).
- 11:00 a.m. **Bruce Geller** - Proteinase activities from new strains of *Lactococcus lactis* subsp. *cremoris* (low-fat cheese project).
- 11:15 a.m. **Floyd Bodyfelt** - Preliminary examination of hazelnut enzymes extracts for flavor enhancement of reduced fat Cheddar cheese (low-fat cheese project).
- 11:30 a.m. **Donald McMahon** - Development of thermophilic cultures for manufacture of low-fat and non-fat Mozzarella cheese (low-fat cheese project).
- 12:00 p.m. GROUP LUNCH
- 1:30 p.m. **LOW-FAT CHEESE PROJECT DISCUSSION**
- 2:30 p.m. **OAC MEETING**
- 3:30 p.m. BREAK
- 4:00 p.m. **OAC MEETING (continued)**
- 5:00 p.m. Adjourn



**WESTERN CENTER FOR DAIRY PROTEIN  
RESEARCH AND TECHNOLOGY**

**OPERATIONAL ADVISORY COMMITTEE**

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**WESTERN CENTER FOR DAIRY PROTEIN  
RESEARCH AND TECHNOLOGY**

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**Project Title:** Influence of preadsorbed protein on adhesion of *Listeria monocytogenes* to dairy food contact surfaces.

**Principal Investigator:** Mark Daeschel, OSU

**Project Title:** The Influence of Preadsorbed Protein on Adhesion of *Listeria monocytogenes* to Dairy Food Contact Surfaces

**Personnel:** M.A. Daeschel, Associate Professor, Department of Food Science and Technology, Oregon State University

J. McGuire, Associate Professor, Departments of Bioresource Engineering and Food Science & Technology, Oregon State University

H. Al-Makhlafi and C.K. Bower, Research Assistants, Department of Food Science and Technology, Oregon State University

**Funding:** Western Center for Dairy Protein Research and Technology, US Agency for International Development, Public Health Service Institutional Grant, and Oregon Agricultural Experiment Station

**Objectives:**

1. record b-lactoglobulin adsorption kinetic data on each of a series of silanized silicon surfaces that have been prepared to exhibit varying degrees of hydrophobic and hydrophilic character;
2. compare the measured adsorption kinetics to that predicted by a simple molecular model of interfacial behavior to obtain a measure of the rate at which conformational changes take place on each surface;
3. prepare adsorbed b-lactoglobulin layers of varying age on each type of surface, and quantify the adsorbed mass of each film;
4. expose the surfaces, with and without preadsorbed films, to *Listeria monocytogenes* in order to document the relationship between characteristics of each protein film and the extent and tenacity of resulting microbial adhesion; and
5. model the extent and tenacity of microbial adhesion as a function of contact surface hydrophobicity, or the nature of the preadsorbed film.

**Results:**

During this last year of the project (93-94) we focused on objectives 4 and 5. Project objectives 1 through 3 were completed during the first and second years of the project and are summarized as follows. The adsorption kinetics exhibited by a-lactalbumin (a-lac), b-casein, and bovine serum albumin (BSA) at hydrophilic and hydrophobic silicon surfaces were recorded with ellipsometry as well, and interpreted with reference to a simple mechanism for irreversible protein adsorption. These additional tests were performed in response to TAC recommendations, and constitute a natural extension of past work that involved measurement of b-lactoglobulin (b-lg) adsorption kinetics on silicon surfaces varying in wettability. With regard to b-lg, a model based on the mechanism described the data very well, enabling interpretation of the kinetic behavior in terms of contact surface hydrophobicity influences on rate constants affecting protein attachment and unfolding at the interface. In particular, both experimental and simulation results indicated that if the process of protein adsorption is resolved into two steps, the first being reversible adsorption defined by kinetic rate constants  $k_1$  and  $k_{-1}$  for attachment and detachment, the second being a conformational change defined by a kinetic rate constant  $s_1$  (resulting in conversion of reversibly adsorbed protein to an irreversibly adsorbed species),  $k_1$  and  $s_1$  increase with increasing solid surface hydrophobicity, while  $k_{-1}$  decreases. Quantitative consideration of possible mass transfer influences on the observed adsorption rates indicated that the experiments were not conducted in a transport-limited regime. In the

present work, a-lac, b-casein, and BSA adsorption kinetics were measured and interpreted with reference to the same model. To date, we have performed a number of kinetic experiments with each of the four proteins, but quantitative conclusions are still tentative. However, although molecularly dissimilar in several ways, differences in surface activity among these proteins at hydrophilic and hydrophobic interfaces can be at least qualitatively explained with reference to molecular flexibility and stability. These have been observed to be very important factors influencing a-lac, b-casein, b-lg and BSA interfacial behavior at air-water interfaces, and a-lac, b-lg and BSA interfacial behavior at solid-water interfaces. b-Casein has a largely unordered structure, but is distinctly amphiphilic; it adsorbed at a greater initial rate and in a greater amount than the other proteins on each type of surface. Results-to-date lead us to infer that it exhibits a higher affinity for hydrophilic surfaces. The globular proteins a-lac and BSA behaved in a manner more similar to that of b-lg at each surface; however, the difference in a-lac adsorption to hydrophilic and hydrophobic surfaces was quite large, with a-lac adsorbing to a much greater extent on hydrophobic surfaces. a-Lac, a small and resilient protein, preliminarily appears to exhibit a higher initial adsorption rate, than does BSA, to hydrophobic surfaces as well. BSA consists of three large domains and nine subdomains. Its surface activity appears largely governed by relatively slow unfolding of one of these domains after adsorption. BSA did not appear to attain a plateau in adsorbed amount after eight hours of contact on either surface, although it did yield a greater adsorbed amount than a-lac in the same time period.

For objectives 4 and 5, experimental protocols were developed to establish consistent quantification of adhered bacterial cells (*L. monocytogenes*). Flow through chambers were employed for reproducible rinsing to establish baseline bacterial cell adhesion to surfaces. Computer driven microscopic Image analysis was used for visualizing and enumerating bacterial cells adhered to surfaces. Our results (In press, Appl. Environ. Micro.) clearly indicated significant differences in the numbers of adhered cells to surfaces with different pre-adsorbed proteins. Briefly, Protein films adsorbed onto surfaces were allowed to contact *L. monocytogenes* for 3 h. After an 8 h protein adsorption time, cell adhesion to surfaces coated with b-lg was greatest followed by surfaces coated with b-casein and a-lactalbumin. Surfaces coated with BSA allowed the lowest adhesion.

The data obtained were interpreted with reference to a simple kinetic model for protein arrival and unfolding as well as molecular properties of each protein. The observed data were found to be consistent with the relative rankings of rate constants defining arrival and unfolding for these proteins.

### **Impact of research to the Dairy Industry**

The ability of proteins to adsorb to food contact surfaces is well recognized. It is possible to capitalize on this phenomenon by specifically allowing antimicrobial or otherwise surface-passivating proteins to adsorb and provide an active deterrent to bacterial adhesion. Antimicrobial proteins are particularly attractive to use because of their known chemical and physical properties, their record of safe and efficacious use and their demonstrated lethality toward *L. monocytogenes*. The wide spectrum of food contact surfaces present in commercial settings presents a challenge to development of generic strategies that prevent adhesion and biofilm formation. Our approach includes quantitative evaluation of protein surface behavior as a function of molecular properties and surface hydrophobicity. Past research supports the importance of both hydrophobic interaction and adsorption competition on formation of an interfacial film. This work will go a long way toward allowing us to optimize noncovalent immobilization of passivating components in order to provide an effective and easily implemented barrier to *Listeria* adhesion.

## Publications:

Al-Makhlafi, H., McGuire, J. and M.A. Daeschel. 1994. The influence of Preadsorbed Milk Proteins on the Adhesion of *Listeria monocytogenes* to hydrophobic and hydrophilic silica surfaces. Appl. Environ. Microbiology. ( In press)

Daeschel, M.A., McGuire, J. and Al-Makhlafi, H. 1992. Antimicrobial activity of nisin adsorbed to hydrophilic and hydrophobic surfaces. J. Food Prot., 55:731

Krisdhasima, V., McGuire, J. and Sproull, R. 1992. Surface hydrophobic influences on b-lactoglobulin adsorption kinetics. J. Colloid Interface Sci., 154:337

Suttiprasit, P., Krisdhasima, V. and McGuire, J. 1992. The surface activity of a-lactalbumin, b-lactoglobulin and bovine serum albumin I. Surface tension measurements with single component and mixed solutions. J. Colloid Interface Sci., 154:316

Suttiprasit, P. and J. McGuire. 1992. The surface activity of a-lactalbumin, b-lactoglobulin and bovine serum albumin II. Some molecular influences on adsorption to hydrophilic and hydrophobic silicon surfaces. J. Colloid Interface Sci., 154:327

**Project Title:** Using a natural process to improve milk quality and extend milk shelf-life through the reduction of lipid oxidation and off-flavor with tocopherol (vitamin E) supplementation to dairy cows.

**Principal Investigator:** Gerald Schelling

**Project Title:** Using a Natural Nutrient Process to Improve Milk Quality and Extend Milk Shelf-Life Through the Reduction in Lipid Oxidation and Off-Flavors with Tocopherol (Vitamin E) Supplementation to Dairy Cows

**Personnel:** Gerald T. Schelling, Department of Animal and Veterinary Science, University of Idaho

Richard A. Roeder, Department of Animal and Veterinary Science, University of Idaho

John Montoure, Department of Food Science and Toxicology, University of Idaho

**Funding:** Western Center for Dairy Protein Research and Technology

**Introduction:**

The oxidation of milk causing off-flavors continues to be a problem in the dairy industry. A recent study of retail milk indicated that up to 38 % of the samples had unacceptable flavor. The role of vitamin E as an antioxidant in milk is recognized. However it is not currently clear what nutritional conditions of dairy cows are important for optimal vitamin E impact upon the milk tocopherol content, and therefore oxidation, of milk. This work is designed to provide information on vitamin E relative to lipid oxidation in milk.

**Objectives:**

The specific objectives of the study are as follows:

1. To evaluate the effect of various chemical and/or administration forms of vitamin E on milk vitamin E content and milk oxidation.
2. To determine the efficiency of gastrointestinal tract absorption and mammary transfer of vitamin E to milk.
3. To determine the effect of higher dietary levels of vitamin A on the vitamin E content of milk and milk oxidation.

**Results:**

To achieve objectives 1 and 2 of this research, an incomplete Latin square design with two periods and 16 lactating Holstein cows were used to evaluate four treatments. The treatments were control (C), 4,000 IU of oral dl-alpha-tocopheryl acetate (ODLT), 4,000 IU of oral micellized d-alpha-tocopherol (ODT), and 4,000 IU of injectable d-alpha-tocopherol (IDT).



Each vitamin E source was administered once as a single dose. The oral treatments were each given as a bolus in a gelatin capsule, and the injectable treatment was delivered into the muscle at two sites with a 3.8 cm needle. Total alpha-tocopherol secretions in milk due to treatments ODLT, ODT and IDT were 13.1, 27.2 and 109.6 mg. The total alpha-tocopherol milk secretion response to the IDT treatment ( $P < .0046$ ) was 3.01 % of the administered dose. The alpha-tocopherol milk secretions due to the oral treatments ODLT and ODT represented .36 and .75 % of the administered doses, with ODT being greater ( $P < .0028$ ) than ODLT.

The rate of milk oxidation was measured over an appropriate time period on milk samples with added ferric sulfate by using the thiobarbituric acid (TBA) procedure. Table 1 shows the decreased oxidation of milk as milk alpha-tocopherol is increased via more available vitamin E forms or administration modes. Relative milk oxidation was significantly ( $P < .01$ ) reduced from 100 % for the control (C) to 82.1, 77.8 and 68.6 % for the ODLT, ODT and IDT treatments respectively. Table 1 also shows the close relationship between blood alpha-tocopherol concentration and milk oxidation.

Table 1.  
Effect of Dietary Vitamin E Sources on Vitamin E Utilization and Milk Oxidation

Vitamin E Source	Blood Vitamin E	Milk Vitamin E	Milk Oxidation
	ug/ml	ug/ml	relative %
C	8.6	.64	100
ODLT	10.2	.72	82
ODT	11.3	.98	78
IDT	23.5	1.73	69

The effect of various levels of dietary vitamin A on milk vitamin E and milk oxidation was studied in an incomplete Latin square design with two periods using 16 mid-lactation Holstein cows to evaluate four treatments. All Cows received 4,000 IU of supplemental dl-alpha-tocopheryl acetate and graded supplemental vitamin A levels of 25,000 (low), 75,000 (medium), 225,000 (high) or 675,000 (very high) IU of vitamin A acetate per head per day. Increasing the dietary vitamin A levels decreased the vitamin E content of both blood and milk. Figure 2 shows the increase in milk oxidation commensurate with the decrease in vitamin E content of the milk caused by high dietary vitamin A levels. When the low vitamin A treatment was set at a relative oxidation of 100 %, the relative oxidation was 125, 119 and 155 % for the medium, high and very high vitamin A treatments respectively.

Table 2.  
Effect of Dietary Vitamin A Levels on Vitamin E Utilization and Milk Oxidation

Vitamin A Treatment	Blood Vitamin E	Milk Vitamin E	Milk Oxidation
IU/day	ug/ml	ug/ml	relative %
25,000	12.4	1.36	100
75,000	12.2	1.31	125
225,000	11.8	1.32	119
675,000	10.1	1.08	155

**Impact of Research:**

The results of these studies will allow for the more knowledgeable formulation of dairy rations to reduce milk oxidation. The impact on formulating dairy rations will be as follows:

1. Relative efficiency data of different chemical forms and/or administration routes will allow for the selection of the most cost effective means of providing vitamin E to achieve certain milk concentrations of vitamin E.
2. The negative impact of high vitamin A levels on vitamin E utilization provides the information needed to make the necessary dietary vitamin E adjustment to maintain certain milk concentrations of vitamin E.

**Publications:**

- Pumphrey, W. M., K. G. DeYonge, J. A. Slusark, M. J. Garber, R. A. Roeder, W. K. Sanchez and G. T. Schelling. 1993. Absorption of various forms of vitamin E from the gastrointestinal tract and transfer across the mammary gland in lactating dairy cows. *J. Anim. Sci* 71:256, Suppl.1.
- Schelling, G. T., R. A. Roeder, M. J. Garber, W. M. Pumphrey and W. K. Sanchez. 1993. The need for increased antioxidant capacity for optimal meat and milk quality. *Proc. Fifteenth International Congress of Nutrition Abstracts* 1:70. Adelaide, Australia.
- Garber, M. J., R. A. Roeder, J. C. Miller, R. P. Richard and G. T. Schelling. 1993. Dose-response effects of vitamin E supplementation on carcass characteristics and storage properties of beef from dairy steers. *J. Anim. Sci.* 71:152, Suppl. 1.
- Schelling, G. T., R. A. Roeder, M. J. Garber and W. M. Pumphrey. 1994. Bioavailability and interaction of vitamin A and vitamin E in ruminants. *J. Nutr.* (in press).

**Project Title:** Function of whey protein and lactose in age  
gelation of UHT-processed milk concentrate - Part  
2.

**Principal Investigator:** Donald McMahon

**Project Title:** Function of whey proteins and lactose in age gelation of ultra-high temperature sterilized milk concentrate, Part 2.

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**Funding:** WESTERN CENTER FOR DAIRY PROTEIN  
RESEARCH AND TECHNOLOGY  
Utah Agricultural Experiment Station  
USDA Agricultural Research Service

**Objectives:**

The mechanism by which age gelation in UHT sterilized milk concentrates occurs is still unknown. There have been many factors implicated and at best an empirical approach is taken to extend shelf life of sterilized milk products. UHT sterilization promotes association between  $\kappa$ -casein and  $\beta$ -lactoglobulin. In milk concentrates the concentration of whey proteins and lactose are increased. Their role in the age gelation process will be studied in this project. The specific objectives of this project are to:

1. Determine the influence of lactose concentration of milk concentrates on age gelation.
2. Determine the fate of  $\beta$ -lactoglobulin during storage of UHT sterilized milk concentrates.
3. Monitor changes in casein micelle structure during storage of UHT sterilized milk concentrates.

**Results:**

**Objective 1.** This was completed in part 1 of this project.

**Objective 2 & 3.**

Using antibodies to  $\beta$ -lactoglobulin,  $\alpha$ -lactalbumin,  $\alpha_{s1}$ -casein,  $\alpha_{s2}$ -casein,  $\beta$ -casein and  $\kappa$ -casein (obtained from J.J. Stastny, University of Illinois College of Medicine), goat ant-mouse IgG conjugated to 10 nm gold, a protocol for immunolabeling of milk samples was established and applied to milk, milk concentrated by ultrafiltration to 3X (volume reduction), and the UF milk heated to 110, 120, 130, and 140°C. The location of these proteins was determined (with the exception of  $\alpha_{s2}$ -casein which did not label). These proteins were then tracked during 12 months storage of the UHT (140°C) milk. A mechanism for age gelation in concentrated milk is proposed.

### **Abstract 1.**

A simple apparatus has been developed for a new "microcube" encapsulation of fluid milk samples in their prefixation preparation for electron microscopy. The new technique is based on making cubic wells in an agar gel layer, filling them with fluid milk samples, and sealing them with another agar gel layer. The individual wells are then separated by cutting from the initial block, providing .5 mm walls around the samples. The embedded material (milk, buttermilk, yogurt, etc.) is fixed, dehydrated, and embedded in a resin for transmission electron microscopy. The procedure is simpler, more versatile, reliable, and reproducible than other encapsulation methods used to prepare similar food samples. Agar gel tubes used in the other methods have several disadvantages such as the need for manual dexterity of the experimenter to make them, and difficulty in sealing the filled capsules properly. Results obtained by the microcube procedure were compared with results obtained by two methods using agar gel tubes and also by mixing a warm agar sol with fluid food samples. This latter method is simpler than agar encapsulation, but shows agar strands in the micrographs of the milk samples. This is particularly undesirable when investigating, for example, intermicellar strands of gelled UHT-sterilized milk concentrates. Microcube encapsulation produces superior quality images of the fluid food structure.

### **Abstract 2.**

A protocol has been established for aldehyde fixation and immunogold labeling of  $\beta$ -lactoglobulin in milk samples, using trichloroacetic acid precipitated milk whey protein from direct ultra-high temperature-sterilized milk retentate (ultrafiltration concentrated 3x by volume reduction). Microcube prefixation encapsulation was used for all samples. Aldehyde degradation of antigen was investigated using enzyme-linked immunosorbent assay (ELISA). Antigenicity of the samples is reduced by both aldehyde fixatives, but paraformaldehyde is less deleterious than glutaraldehyde. Paraformaldehyde (4%) with fixation times of 2 h, 3 h, 4 h, and 5 h and glutaraldehyde (1.5%) with fixation times of .5 h, 1 h, 2 h, and 3 h were investigated to determine the optimal fixation time for maximum protein antigenicity. The 4 h fixation with paraformaldehyde (4%) and 1 h fixation with glutaraldehyde (1.5%) best preserves antigenicity while providing adequate fixation of the protein. The paraformaldehyde fixation results in better sections at labeling. LR White resin polymerized at 50°C was satisfactory for the embedding of samples. Teleosteam fish gelatin (.1%) with normal goat serum (.1%) in 20 mM Tris buffer without bovine serum albumin proved to be an adequate blocking agent. The protocol localizes  $\beta$ -lactoglobulin in gelled and fresh UHT-sterilized UF milk retentate.

### **Abstract 3.**

Samples of anti- $\beta$ -lactoglobulin and anti- $\alpha$ -lactalbumin are used in Western blot analyses and immunolocalization studies of whey at various stages of preparation from fresh samples to ultra-high temperature-sterilized samples. Western blot analyses of the samples show that the antibodies are specific for the native as well as the complexed forms of  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin. Immunolocalization studies show that the native form of the whey proteins is more susceptible than the complexed form to leaching through the fixation, dehydration, and embedding processes of electron microscopy preparation. Most of the labeling observed in immunolocalization analyses of milk samples for  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin, identifies these proteins complexed with each other or with other milk proteins.

### **Abstract 4.**

Immunolocalization techniques are employed to elucidate the positions of  $\beta$ -lactoglobulin,  $\alpha$ -lactalbumin,  $\alpha_{s1}$ -casein,  $\alpha_{s2}$ -casein,  $\beta$ -casein, and  $\kappa$ -casein in milk at various stages of treatment. These treatments comprise fresh whole milk, skim milk, pasteurized milk, ultrafiltered milk, and direct and indirect sterilized (110, 120, 130, and 140°C) milk.

Heating of milk through pasteurization and UHT sterilization affects the distribution and alters the conformational state of some milk proteins. This is more pronounced with  $\beta$ -lactoglobulin where interaction with whey and micellar casein protein is observed as a function of processing temperature.  $\alpha$ -Lactalbumin and  $\kappa$ -casein show a weaker response.  $\alpha_{s1}$ -Casein and  $\beta$ -casein show heavy specific labeling concentrated on the micelles, but no effect of heating on protein distribution is evident with these proteins.  $\alpha_{s2}$ -Casein did not respond to these immunolocalization procedures.

### **Abstract 5.**

Immunolocalization techniques are employed to elucidate the positions of  $\beta$ -lactoglobulin,  $\alpha_{s1}$ -casein,  $\beta$ -casein, and  $\kappa$ -casein in stored UHT-sterilized UF milk retentate from the day of preparation through to age gelation. The milk retentate had been stored at room temperature and sampling done bimonthly. Denatured  $\beta$ -lactoglobulin complexes on the micellar surface during UHT preparation and moves back to the intermicellar spaces during prolonged storage. The labeling of  $\kappa$ -casein is negligible just after UHT preparation, but begins to increase with storage time forming linear patterns within the intermicellar matrix.  $\alpha_{s1}$ -Casein and  $\beta$ -casein label heavily throughout the experiment. Labeling for these two casein moieties appear very specific for the micelles

just after UHT preparation until ~10 months of storage. At this time the labeling spills into the intermicellar matrix, but still shows high specificity for portions of the micelles. Much of the structural integrity of the micelle is retained in the coagulum. Labeling for  $\alpha$ -lactalbumin and  $\alpha_{s2}$ -casein was slight and indeterminate.

A mechanism of age gelation of UHT-sterilized UF-concentrated milk is proposed. The loss of the  $\beta$ -lactoglobulin- $\kappa$ -casein complex from the micelles to the serum exposes the calcium-insoluble micellar  $\alpha_{s1}$ -casein and  $\beta$ -casein to the serum calcium. This would reduce micelle stability and promote coalescence of the micellar proteins, leading to coagulation. The tendrillar appendages appear to be the  $\beta$ -lactoglobulin- $\kappa$ -casein complex which entraps the micellar residues at gelation.

#### **Impact of Research:**

A mechanism for the understanding of age gelation in concentrated milk has been proposed. This may allow those in the dairy industry who plan to produce such a product to know the limitations of the process and how it can be optimized.

#### **Publications:**

- ✓ Alleyne, M.C., McMahon, D.J., Youssef, N.N. and S. Hekmat. 1993. An apparatus for a new microcube encapsulation of fluid milk in preparation for transmission electron microscopy. *Food Struct.* 12:21-30.
- Alleyne, M.C., D.J. McMahon, W.R. McManus, and L.C. Burgess. 1994. Characterization of immunolocalization of  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin in milk and whey samples. *Food Struct.* (Submitted for publication).
- Alleyne, M.C., D.J. McMahon and W.R. McManus. 1994. Comparing antigenicity of  $\beta$ -lactoglobulin in UHT-sterilized milk retentate and whey using different modes of aldehyde fixation. *Food Struct.* (Submitted for publication).
- Alleyne, M.C. and D.J. McMahon. 1994. Immunolocalization of caseins and whey proteins in milk at various stages of treatment from fresh whole milk to ultra-high temperature-sterilized ultrafiltered (3X) milk concentrate. *J. Dairy Science* (Submitted for publication).
- Alleyne, M.C. and D.J. McMahon. 1994. Immunolocalization of caseins and whey proteins in ultra-high temperature-sterilized ultrafiltered (3X) milk concentrate stored at room temperature until gelation. *J. Dairy Science* (In preparation).

**Project Title:** Effects of iron fortification on chemical, physical, microbiological and nutritional properties of yogurt.

**Principal Investigator:** Donald McMahon



**Project Title:** Effects of Iron Fortification on Chemical, Physical and Microbiological and Nutritional Properties of Yogurt

**Personnel:** Donald J. McMahon  
Sharareh Hekmat  
Mrudula Kalpalathika  
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Nutrition & Food Sciences Dept., Utah State University  
William R. McManus  
Biology Dept., Utah State University

**Funding:** WESTERN CENTER FOR DAIRY PROTEIN  
RESEARCH AND TECHNOLOGY  
Utah Agricultural Experiment Station

**Objectives:**

- A. Produce low fat and non fat iron-fortified yogurt using FeCl<sub>3</sub>, Fe-Casein and Fe-Whey Protein complexes as the iron sources.
- B. Determine growth and viability of *Lactobacillus delbruekii* ssp. *bulgaricus* and *Streptococcus thermophilus* in iron-fortified yogurt.
- C. Determine iron binding sites using Electron Spectroscopy Imaging.
- D. Determine the best procedure for making high-quality iron fortified yogurt.

**Results:**

**Effect of iron fortification of yogurt on its manufacture, growth of lactic acid and spoilage bacteria, and lipid oxidation.**

Low-fat (2%) and non-fat iron fortified yogurt were made with three sources of iron, FeCl<sub>3</sub>, Fe complexed with casein, and Fe complexed with whey protein, at three levels (10, 20, 40 mg/kg). The ferrozine assay and thiobarbituric acid test were used to quantitate iron content and lipid oxidation respectively over one month of storage of the yogurt at 4°C. Survival of *Lactobacillus delbruekii* ssp. *bulgaricus* and *Streptococcus thermophilus* were monitored using MRS (pH 5.4) and M17 media respectively. To determine whether presence of iron would promote growth of pathogenic or spoilage microorganisms, regular skim yogurt mix and iron fortified yogurt mix were inoculated with *Escherichia coli* DH5a and *Pseudomonas fluorescens* (ATCC 31732) to 10<sup>3</sup> and 10<sup>5</sup> cfu/ml of yogurt.

Iron fortification had no effect on the rate of fermentation by the lactic cultures. After fermentation the lactic acid bacterial counts were 10<sup>8</sup> cfu/ml for both *L. delbruekii* ssp. *bulgaricus* and *S. thermophilus* and these decreased only slightly during one month of

storage. The bacterial counts for *P. fluorescens* and *E. coli* decreased to less than  $10^1$  cfu/ml at day 1 and 15 respectively. There were no significant increases in oxidation levels between iron fortified yogurt and non-fortified yogurt ( $P=.05$ ). No differences in flavor between the iron-fortified yogurt and non-fortified yogurt were detected in sensory analysis using an untrained panel and all samples were strongly liked. Our study shows that yogurt is a suitable vehicle for delivering iron to consumers.

#### **Binding of iron to casein and whey proteins and in Fe-fortified yogurt.**

Iron binding affinity of casein and whey protein was studied by fortifying skim milk with 10 mg Fe/100 ml of milk and adjusting its pH to 6.7, 6.2, 5.8, 5.3, 4.5, and 4.0. These samples were ultracentrifuged and casein pellets and whey solutions were collected, digested with nitric acid, and then analyzed for iron by inductively coupled plasma (ICP) spectroscopy. SDS-PAGE was used to determine whey protein composition over this pH range. To further study iron binding of casein at low pH, skim yogurt was fortified with  $\text{FeCl}_3$ , Fe complexed with casein, and Fe complexed with whey protein. Yogurt was freeze-dried on carbon grids and then examined without heavy metal staining by transmission electron microscopy. Electron spectroscopic imaging (ESI) was then used to acquire elemental images and produce a map of Fe distribution in the samples.

As pH decreased, Fe binding capacity of casein increased while that of whey protein decreased. SDS-PAGE of whey showed clear bands for  $\alpha_s$ -casein, b-casein and k-casein at pH's 6.7, 6.2, 5.8 and 5.3. These bands were missing at pH's 4.5 and 4, indicating that at higher pH's, some casein was retained in the supernatant after ultracentrifuging. The Fe measured in the whey was most likely bound to this casein. Using ESI it was observed that when yogurt was fortified with Fe-casein, the Fe remained bound to the casein and was distributed throughout the micelles; with Fe-whey protein, it was distributed throughout the non-micellar portion of the yogurt; with fortification by  $\text{FeCl}_3$ , virtually all the Fe was observed to be bound preferentially to the casein and was located within the casein micelles.

#### **Impact of Research:**

Increasing the resolution available when using scanning electron microscopy to study the microstructure of mozzarella cheese will allow this technique to be used effectively in studying its microstructure. Developing a method to more appropriately measure stretch of melted mozzarella cheese will allow research results to be more directly applied to industry practices in the manufacture of mozzarella cheese for use on pizzas.

**Publications:**

Hekmat, S., Reddy, M.I., McManus, W.R., and D.J. McMahon. 1994. Binding of iron to casein and whey proteins and in Fe-fortified yogurt. 89th American Dairy Science Association Meeting, *J. Dairy Sci.* 77(Supp. 1):35

Hekmat, S., Wiemer, B., and D.J. McMahon. 1994. Effect of iron fortification of yogurt on its manufacture, growth of lactic acid and spoilage bacteria, and lipid oxidation. 89th American Dairy Science Association Meeting, *J. Dairy Sci.* 77(Supp. 1):19

**Project Title:** Interactions between milk proteins, starter cultures  
and hydrocolloidal milk fat replacers.

**Principal Investigator:** Bart Weimer

**Project Title:** Interactions between milk proteins, starter cultures and hydrocolloidal milk fat replacers.

**Personnel:** Bart Weimer, Dept. of Nutrition and Food Sciences, Utah State University  
Donald McMahon, Dept. of Nutrition and Food Sciences, Utah State University  
Mike LeFevre, initially Utah State University, currently Kerry Foods  
Chris Allyne, Dept. of Nutrition and Food Sciences, Utah State University  
Christina Beer, Dept. of Nutrition and Food Sciences, Utah State University

### **Introduction**

Health experts are urging people to reduce the amount of fat consumed in their diet. Consumers are demanding more lowfat and non-fat food products. Many food ingredient companies have responded by developing fat substitutes. Simplese® (The NutraSweet Co.) and Stellar™ (Staley Manufacturing Co.) are just two of these. To replace milk fat, non-fat substances (such as starch or protein) should mimic milk fat globules. This means the starch or protein fat replacer should have a similar size distribution and be able to interact with milk proteins the way fat globules do. Different companies have developed fat replacers for segments of the food industry. Though some products do not target milk fat replacement, some companies claim their product can act as milk fat replacers in dairy products. The lowfat and non-fat food market is exploding. We will examine how fat replacers interact with milk proteins to mimic milk fat globules.

Addition of fat replacers to milk creates a new dimension to the microbiology of fermented dairy products. Interactions between fat replacers and starter cultures are unknown. Fat replacers offer starter bacteria new sources of growth nutrients in milk. Starter cultures' growth and acid production will be influenced by protein- and starch-based fat replacers. Functionality of fat replacers will be effected by the proteinases, peptidases and metabolic end-products of lactic acid bacteria. We will investigate the interaction between starter cultures and fat replacers and the effect of each during cheese making.

### **Objectives**

1. Examine coagulation, protein binding, and stability of milk proteins in the presence of fat replacers.
2. Stability of fat replacers in dairy systems during growth of lactic starter cultures.

### **Results**

Research to date has focused on objective 2. Experiments were begun for objective 1 when the Post-doctoral fellow left the project. Another Post-doctoral fellow was found recently and work to complete objective 1 is underway.

Forty two starter cultures were screened for their ability to produce acid in milk and media containing different fat replacers which demonstrated starter cultures can degrade fat replacers to produce acid during growth. Data suggests acid production could be difficult to control with added fat replacer and is dependent on the strain and fat replacer used during manufacture as low levels of enzymes activity that degrade the fat replacers was found. Strain characterization was done using colorimetry for individual glucolytic, peptidolytic enzymes, and the Biolog system as a method to predict the rate at which strains degrade fat replacers. The Biolog system was successfully used to screen strains for glucolytic enzymes, but did not reveal the rate of degradation. Colorimetric assays for these enzymes indicated the rate of hydrolysis of starch-based fat replacers is relatively low but is a significant source of acid. Hydrolysis of protein-based fat replacers was less common in the starters tested, but if present significantly increases acid production and cell growth in milk. Slendid was the only fat replacer tested that was not hydrolyzed and inhibited growth of starter cultures.

Coagulation characteristics of milk containing fat replacers at various levels varied with each fat replacer added and is currently being evaluated. Preliminary results indicate bacteria are associated with the fat replacers in cheese. These data taken together suggest that cheese made with fat replacers be significantly different from the full fat version and the acid production rate during manufacture will be a source of variation. Distribution of the fat replacers also vary according to the type of replacer used.

### **Significance to the Dairy Industry**

Use of fat replacers is increasing in the dairy industry. Determination of the chemical interactions in cheese is required for their successful use in flavorful lower fat cheeses. This work highlights that protein-based fat replacer behave similarly, but different from starch-based fat replacers. Use of starch-based fat replacers tend to have more degradation due to microbial conversion of the sugars to lactic acid which impacts cheese making.

### **Publications**

none to date

### **Abstracts**

LeFevre, M. and B. Weimer. 1993. Metabolic characterization of low fat Cheddar cheese starter cultures. *J. Dairy Sci.* 76 (supp 1):130.

**Project Title:** Production of extracellular proteases of  
*Brevibacterium linens* for use in low-fat cheese.

**Principal Investigator:** Bart Weimer

**Project Title:** Production of extracellular proteases of *Brevibacterium linens* for use in low-fat cheese

**Personnel:** Bart Weimer, Dept. of Nutrition and Food Sciences, Utah State University  
Ummadi Madhavi, Dept. of Nutrition and Food Sciences, Utah State University  
Benjamin Dias, Dept. of Nutrition and Food Sciences, Utah State University

### Introduction

Consumers are demanding more lowfat and nonfat foods. Many food ingredient companies have responded by developing fat substitutes made from protein, starch, and pectin. The dairy industry has little experience using fat replacers in fermented milk products.

Addition of fat replacers creates a new dimension to the microbiology of fermented dairy products. Interactions between fat replacers and dairy cultures are unknown. Functionality of fat replacers will be effected by the proteinases, peptidases, and other metabolites the bacteria used during cheese making.

Lack of flavor development and hard body in reduced fat dairy products is a common problem. Highly proteolytic organisms, like *Brevibacterium linens*, have been used to accelerate the ripening of full fat cheese. They sometimes cause too much proteolysis which result in strong flavored and soft bodied full fat cheeses. Use of this organism in lowfat cheese may produce a product which has an acceptable level of flavor and body. We will investigate the use of a whey-based medium to produce enzymes that increase flavor development and body in Cheddar cheese made with fat replacers.

### Objectives

1. Develop a whey-based medium for the production of extracellular proteases by *Brevibacterium linens*.
2. Determine the interaction of the rate of proteolysis from proteases produced by *Brevibacterium linens* in the presence of fat replacers and coagulating enzymes in milk.
3. Develop processing parameters required to produce Cheddar cheese with acceptable flavor and body using milk fat replacers and proteases from *Brevibacterium linens*.

### Results

Significant effort has been placed on objective 3 while objectives 1 and 2 are currently underway. Proteolysis in cheese made with *B. linens* differs depending on the strain used in manufacture. Fat replacer degradation occurs for protein- and starch-based fat replacers in laboratory conditions. These data indicate that use of *B. linens* as a flavor adjunct is possible, but care must be used for off-flavor generation during aging. Biochemical characterization



supports the observations made for cheese with each strain tested exhibiting different glucolytic, proteolytic, peptidolytic, and lipolytic activity in culture.

Initial results indicate media significantly influences protease production and activity. Media formulations are currently being evaluated. Peptidase and lipase activity is also influenced in a strain specific manner. Studies to determine the optimum media formulation to enhance desirable traits and minimization of undesirable traits are underway.

### **Significance to the Dairy Industry**

Use of *B. linens* has been shown to be an acceptable flavor adjunct for use in low fat cheese. Determination of cheese making and growth conditions will lead to recommendations for cheese making to increase desirable flavors and minimization of undesirable off-flavors with and without fat replacers. Currently, work continues to define specific enzyme systems that need to be increased for enhance flavor in lower fat cheese.

### **Publications**

none to date

### **Abstracts**

Dias, B. and B. Weimer. 1993. Peptidase activity of adjunct lactobacilli used in the manufacture of low fat Cheddar cheese. *J. Dairy Sci.* 76 (supp 1):122.

Dias, B., U. Madhavi, and B. Weimer. 1994. Characterization and comparison of peptidase and lipase activity among *Lactococcus lactis* ssp. *cremoris*, *Lactobacillus helveticus*, and *Brevibacterium linens*. American Society for Microbiology Annual Meeting, Las Vegas.

**Project Title:** Bacteriophage-resistance gene replacement in  
*Lactococcus lactis*.

**Principal Investigator:** Bruce Geller

**Title:** Bacteriophage-resistance gene replacement in *Lactococcus lactis*

**Personnel:** Bruce Geller  
Department of Microbiology  
Oregon State University

**Funding:** Western Center for Dairy Protein Research and Technology

#### Introduction

My lab has cloned and sequenced a gene from *Lactococcus lactis*, called pip, which is required for bacteriophage infection. We have tested approximately 15 phages that attack our test strain (*L. lactis* ssp. *lactis* C2), and all but one phage (sk1) is completely resistant to the strain with a mutated pip. In addition, we have evidence that pip is required for phage infection of other strains.

The project is beginning its third year, and is in the process of creating better phage-resistant strains than are currently available. This is being done by exchanging a mutated copy of pip with the normal copy of pip in the phage-sensitive strain. The methods we are using for genetic exchange will allow the introduction of the mutated pip into many different strains of *L. lactis*. This should make it easy to convert any existing strain to a phage-resistant strain by exchanging pip.

#### Specific Objectives

The primary objective of the proposed research is: To replace the wild-type chromosomal pip gene with a mutated copy, in order to create a stable, phage-resistant strain.

Strategically, the intermediate aims necessary to attain the primary objective are:

- a. To make in vitro mutations in the cloned gene required for phage infections.
- b. To insert the mutated copies of pip into the chromosome and exchange it with the wild-type gene.
- c. To test the different mutants for viability, phage resistance, and

growth characteristics favorable to cheese-making.

### Significance

Phage infection of cheese and other dairy fermentations is the most significant cause of ruined fermented milk products. Because nearly 30 % of the raw milk produced in the United States is used to make cheese, even a small percentage of the milk ruined by phage contamination represents a large quantity of lost milk, as well as lost profits and higher consumer costs. Often the product of failed cheese fermentations must be disposed of entirely, or sold at a small fraction of what high quality products command. Reduction or elimination of this problem would decrease the waste of milk, and through economic means, would increase the consumption by humans.

The proposed research would create new strains of lactic bacteria for starter cultures that are more phage-resistant than currently available. This strain improvement project differs significantly from those in the past, in that it proposes to use molecular genetic engineering techniques to introduce mutational changes not practically possible by conventional strain improvement technology. It must be emphasized that these genetically engineered strains would contain no foreign DNA, no additional DNA of any kind, and would be food-grade organisms according to current FDA rules.

The proposed research plans to take what we have learned from our basic research on phage infections and apply it to the development of new strains. The new strains will be tested for their growth characteristics necessary to make cheese. This testing includes the effects of the new strains on milk proteins, such as the casein proteolytic activities and coagulation times. In this way, the aims of the proposed research are very much in keeping with the focus of the Western Center for Dairy Protein Research and Technology.

### Results

Since our last report on January 17, 1993, we have attempted to replace pip in LM2301 with a transposon-mutated copy. We have screened hundreds of potential isolates, and found at least 10 that have the correct phenotype. Our putative pip-replaced strains are phage c2 resistant and phage sk1 sensitive, indicating that the strains are not contaminants, but derived from LM2301. However, genomic Southern blots from the putative pip-replaced strains did not confirm that pip was replaced. In fact, the Southern blots revealed a wild-type size pip. This is particularly difficult to reconcile, because the putative pip-replaced strains are erythromycin sensitive (this was the selectable marker on the replacement plasmid), and were never selected for phage resistance. While one might suspect a contaminant, this is inconsistent with the phage sk1 sensitivity of the isolates.

Since June 1, I have been working full time on this project, in order to resolve the conflicting data. At this time it is still unclear if the putative pip-replaced strains have a transposon-mutated copy of pip in the chromosome. I am currently developing a polymerase chain reaction-based assay to screen the

isolates, which will be much less labor intensive than genomic Southern blots.

#### Deliverables

For FY95 (07\01\94-06\30\95), the deliverables are:

1. A "food-grade", mutated copy of pip, that can be used to integrate into the chromosome of *Lactococcus lactis* and replace the wild-type copy of pip, without leaving any foreign DNA in the chromosome.
2. A strain with the "food-grade", mutated copy of pip inserted into the chromosome and substituted for the wild-type copy.
3. A test of the pip-substituted strain described above. This will include tests of phage resistance and other favorable cheese-making characteristics.

**Project Title:** Development of high protein low-fat fermented foods from yogurt cheese.

**Principal Investigator:** Conly Hansen

**Project Title:** Development of high protein low fat fermented dairy foods from yogurt-cheese.

**Personnel:** Conly L. Hansen, Professor, Dept. of Nutrition and Food Sciences, Utah State University

Yehia A. El-Samragy, Visiting Professor, Dept. of Nutrition and Food Sciences, Utah State University

**Funding:** Western Center for Dairy Protein Research and Technology and Utah Agricultural Experimental Station

**Objectives:**

The main objective of this research project is to develop a process to use yogurt-cheese manufactured from low fat milk retentate as the principal base ingredient to produce new fermented dairy foods. The new products will be characterized with a high protein and low fat content. The possibilities of the fortification of the new products with different additives such as flavors, fruits, vegetables, etc., will be emphasized. This will increase the consumer acceptance to this product and will meet a wide range of nutritional and organoleptic qualities being demanded by different types of consumers according to age, sex, health, and national origin.

1. Develop high protein, low fat, yogurt-cheese from skim milk retentate and do a sensory evaluation on the product.
2. Create new dairy products using yogurt-cheese as a dairy base ingredient and do a sensory evaluation on the new food products.
3. Evaluate the chemical, microbiological and sensory changes in the new dairy foods during storage.

**Results:**

Table 1 indicates the gross composition of soft cheese like product and Cheddar cheese like spread.

The firmness and water-holding capacity of soft cheese like product were close to that determined for Cheddar cheese like spread (data not shown). The total solids content was within a very narrow range of 21.81 to 21.84%, respectively (Table 1).

Some preliminary trials were carried out to organoleptically evaluate the new product in a plain form called yogurt cheese. Taste panelists seemed to get confused because they directed their minds to yogurt more than cheese or something in between yogurt and cheese. We have got a general comment that they want this product

in a flavored form more than in a plain form. This feed back made us change our way of presenting this product to call it soft cheese like product when made in a plain form. Also, we presented it a flavored form using natural Cheddar cheese flavor and a yellow food grade color and called it Cheddar cheese like spread.

Data presented in Tables 2 and 3 summarize the response of the panelists to the two forms of this new fermented dairy food. The scores of the panelists for soft cheese like product were in the positive side for appearance (74%), flavor (70%), richness (66%), spreadability (58%) and overall acceptability (72%) (Table 3). The scores for the Cheddar cheese like product were in the positive side also, for appearance (70%), richness (54%), spreadability (54%), flavor (56%) and overall acceptability (58%) (Table 3). The score for the overall acceptability of the Cheddar cheese like spread was less than the soft cheese like product. This might be explained from the fact that the American consumer used to have the Cheddar cheese in a hard form with flavor ranging from mild to extra sharp. Perhaps using real pieces of fruit or vegetables along with flavor will be more acceptable to the consumer.

#### Significance:

This project will develop a method to manufacture from ultrafiltered skim and/or low fat milk a "yogurt-cheese" with a high protein and non-fat or low fat content. The plain form of yogurt-cheese will be flavored and/or have fruits added to produce a new dairy food that can be eaten alone as a healthy dairy food or served in many ways such as a salad dressing. This product should be well received by consumers because it will meet a wide range of nutritional and organoleptic qualities being requested by consumers of all ages, health status, sex and cultures.

The overall results of this proposal will increase milk usage by opening new markets for dairy products with unique properties which will lead to increased profits for the dairy producer.

#### Deliverable

1. The plain form of this high protein, low fat fermented dairy product manufactured from ultrafiltered low fat milk can be used as a dairy ingredient.
2. It can be Cheddar cheese flavored as was done in the current work or have fruits, vegetables or spices added to produce other new dairy foods, as we are working on now.
3. These can be eaten alone or as healthy dairy foods or served in many ways such as a salad dressing.

We think that this product should be well received by consumers because it will meet a wide range of nutritional and organoleptic



qualities being requested by consumers of all ages, health status, sex and cultures.

**Publications:**

El-Samragy, Y. A. and C. L. Hansen. 1994.  
Sensory Characteristics, Physical Properties and Composition of a New, High Protein, Low Fat, Fermented Dairy Food.  
Abstract. Presented at the 89<sup>th</sup> Annual Meeting of the American Dairy Science Association (ADSA), Minneapolis, Minnesota, July 1994.

**Table 1: Gross composition of soft cheese like product and Cheddar cheese like spread.**

Item	Soft cheese like product	Cheddar spread like spread
Total solids(%)	21.81	21.84
Protein (%)	11.39	11.30
Fat (%)	3.45	3.53
Lactose (%)	4.64	4.72
Ash (%)	1.69	1.65
Salt (%)	0.64	0.64
Titratable acidity (%)	1.30	1.34

**Table 2: Panelists score of sensory evaluation of soft cheese like product.**

Property	Like very much		Like moderately		Like slightly		Dislike slightly		Dislike moderately		Dislike very much		Total	
	No	%	No	%	No	%	No	%	No	%	No	%	No	%
Appearance	1	2	17	34	19	38	12	24	1	2	0	0	50	100
Flavor	1	2	17	34	17	34	12	24	3	6	0	0	50	100
Richness	0	0	15	30	18	36	12	24	5	10	0	0	50	100
Spreadability	0	0	8	16	21	42	14	28	7	14	0	0	50	100
Overall acceptability	0	0	15	30	21	42	7	14	7	14	0	0	50	100

**Table 3: Panelists score of sensory evaluation of Cheddar cheese like spread.**

Property	Like very much		Like moderately		Like slightly		Dislike slightly		Dislike moderately		Dislike very much		Total	
	No	%	No	%	No	%	No	%	No	%	No	%	No	%
Appearance	6	12	20	40	14	28	8	16	2	4	0	0	0	100
Flavor	2	4	12	24	14	28	13	26	6	12	3	6	0	100
Richness	2	4	5	10	20	40	13	26	9	18	1	2	0	100
Spreadability	4	8	11	22	12	24	17	34	5	10	1	2	0	100
Overall acceptability	1	2	19	38	9	18	8	16	12	24	1	2	0	100

**Project Title:** Extrusion processing of milk proteins.

**Principal Investigator:** Conly Hansen

July 28, 1994

## **Extrusion of skim milk protein**

Report to the western dairy foods research center

Conly L Hansen, Principal Investigator

### **INTRODUCTION**

Extrusion processing has found widespread application in the food industry. It's used in restructuring proteinaceous and starchy materials into a variety of products such as: breakfast cereals, noodles and pasta, snack foods, confectionery, pet foods, and texturized proteins.

Consumers are becoming increasingly aware of the nutritional importance of proteins, vitamins, and minerals. New products may require the addition of selected sources of proteins. The challenge, therefore, lies in fabricating foods that combine nutrition, convenience and desirable sensory attributes. The use of some dairy commodities in extruded products has been shown to affect desirable qualities such as flavor, texture, appearance, and nutritional content. These dairy products include whole milk powder, casein powder, and whey proteins. However, incorporation of skim milk powder into extruded food products lacks sufficient research.

### **OBJECTIVES**

1. To study the effects of moisture content, protein content lactose/protein ratio, and feed rate on the physical, functional, and microstructural attributes of extruded corn starch-skim milk powder blends.
2. To determine extrusion conditions most likely to produce extrudates with properties suitable for a snack type product.

### **RESULTS**

#### **Expansion Ratio (ER)**

Moisture content had a significant linear and quadratic effect on ER and also a significant interaction effect with feed rate.

ER decreased with increase in moisture content until 26%-27% moisture content, then increased slightly at high moisture content. At low moisture content, ER decreased slightly at higher feed rates, but at high moisture content the trend was reversed.

#### **Product Temperature**

Product temperature (PT) was influenced by all control variables except feed rate (Table 2). PT decreased with increased moisture and protein content (Figure 3), and with increase in lactose/protein ratio. There were also significant interactions between moisture content

and protein content, and between lactose/protein ratio and feed rate.

#### **Bulk Density**

Moisture content was the only control variable affecting bulk density (BD), although protein content had a significant second order term.

#### **Color**

Moisture content was the dominating variable, having significant linear and quadratic effects and an interaction effect with feed rate. Protein content also had a significant linear effect.

Color decreased with increase in moisture until 26%-27% moisture content and then increased slightly at high moisture content. Color increased with increase in protein content, and was maximum at low moisture content and low feed rate.

#### **Shear Stress**

Moisture and protein content were influencing variables on shear stress, both having significant linear effects. Shear stress increased with increase in moisture content, and decreased with increase in protein content.

#### **Viscosity**

Viscosity increased with increase in moisture content up to 28% moisture, then decreased at very high moisture content. Viscosity was maximum at 26%-28% moisture content with low protein content, and at 26%-28% moisture content with low lactose/protein ratio. Viscosity decreased with increase in protein content and with increase in lactose/protein ratio. There was also a significant interaction effect between protein content and lactose/protein ratio.

#### **Water Absorption Index (WAI)**

WAI increased with increase in moisture content until 26%-28% moisture, then decreased at high moisture content. WAI decreased with increase in protein content, and decreased with increase in lactose/protein ratio. The effect was pronounced at high moisture content. There were significant interaction effects between moisture content and feed rate, and between moisture content and lactose/protein ratio. WAI was maximum at 26%-28% moisture content with high feed rate, and at 26%-28% moisture content with low lactose/protein ratio.

#### **Model Adequacy**

A quadratic model of the form given below expressed the response variables in terms of the control variables.

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{14}x_1x_4 + b_{23}x_2x_3 + b_{24}x_2x_4 + b_{34}x_3x_4 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{44}x_4^2$$

where

y denotes response variable,

$x_1$  denotes moisture content,

$x_2$  denotes protein content,

$x_3$  denotes lactose/protein ratio,

$x_4$  denotes feed rate, and

$b_i$  and  $b_{ij}$  denote regression coefficients.

### **Optimum Extrusion Conditions**

Optimum extrusion conditions were determined for a snack type product. The response variables were either maximized or minimized. The optimum extrusion conditions for each response variable were different. Because ER, BD and shear stress were observed to be correlated to each other, these properties along with color, were chosen to determine conditions for a snack type product. The combined set of extrusion conditions included: 20% moisture content, 16% protein content, 0.347 lactose/protein ratio, and 55.0 gm/min feed rate.

### **SIGNIFICANCE**

The demand for convenience-type foods that combine nutrition with desirable sensory attributes is increasing rapidly. American consumers are increasingly aware of the nutritional importance of proteins, vitamins and minerals, and are avoiding foods containing excess calories and saturated fats. Production of skim milk protein is an established segment of the dairy industry. But foods without acceptable appearance, flavor and texture will not be successful, regardless of nutritional quality. The challenge, therefore, lies in fabricating foods that combine nutrition, desirable sensory attributes and convenience. Extrusion processing offers a unique method of producing nutritious foods that will be readily accepted. Whey proteins can be restructured by extrusion for use in fabricated food products. This versatile method has found widespread application in the food industry for restructuring proteinaceous and starchy materials into a variety of fabricated, cooked and shaped products of varying texture. The extrusion process when developed will expand the utilization of milk products in food.

Not much research has been devoted to explaining what underlying molecular occurrences during extrusion cause the observed functional property changes of dairy macromolecules. Also, research specifically designed to modify milk proteins to lend itself more readily for inclusion in new food products is lacking. This research will help open the way for more applied research in extruded dairy product development.

**Project Title:** Rheology and microstructure of Mozzarella cheese.

**Principal Investigator:** Donald McMahon

**Project Title:** Rheology and Microstructure of Mozzarella Cheese

**Personnel:** Donald J. McMahon  
Craig J. Oberg  
Robert Fife  
Nutrition & Food Sciences Dept., Utah State University  
William R. McManus  
Biology Dept., Utah State University

**Funding:** WESTERN CENTER FOR DAIRY PROTEIN  
RESEARCH AND TECHNOLOGY  
Utah Agricultural Experiment Station

**Objectives:**

It is well known that the stretch and melt characteristics of Mozzarella cheese are important aspects of its appeal to consumers. Although there have been a number of recent investigations of the factors that affect these properties, our knowledge of why Mozzarella cheese stretches and melts is still very limited.

An understanding of the process of cheese melting would enable us not only to control the production of conventional Mozzarella cheese but also develop new cheese products designed to satisfy consumer demands for lowfat cheeses. The objectives of this project are to:

- A. Develop rheological and electron microscopic techniques for the measurement the melting and stretching phenomena of cheese.
- B. Study the protein-fat interactions using the techniques developed above to determine how stretch and melt properties are related.

**Results:**

A metal impregnation technique using tannin-ferrocyanide-osmium tetroxide to impart thermal conductivity to samples destined for scanning electron microscopy was adapted for use with mozzarella cheese. Images were obtained at low and high magnifications having superior characteristics, with minimal edge effects, charging, thermal drift, and decoration. High magnification images were recorded with 3 nm resolution (which approaches the resolution limit of ultra-thin sectioned biological materials (2.5 nm). This combination of metal impregnation, ultra fine iridium metal coating and low voltage field emission SEM yielded images of cheese surpassing any previously published work.

Melt and stretch tests have been modified so that measurements can be made on mozzarella cheese containing less fat than part skim mozzarella cheese. It has also been shown that the state of moisture in mozzarella changes as fat is removed.



**Impact of Research:**

Increasing the resolution available when using scanning electron microscopy to study the microstructure of mozzarella cheese will allow this technique to be used effectively in studying its microstructure. Developing a method to more appropriately measure stretch of melted mozzarella cheese will allow research results to be more directly applied to industry practices in the manufacture of mozzarella cheese for use on pizzas.

**Publications:**

McManus, W., McMahon, D.J. and C.J. Oberg. 1993. High-resolution scanning electron microscopy of milk products: A new sample preparation procedure. *Food Struct.* 12:475-482.

Oberg, C.J., W. McManus, and D.J. McMahon. 1993. Microstructure of Mozzarella cheese during manufacture. *Food Structure* 12:251-258.

McMahon, D.J., Oberg C., and W. McManus. 1993. Functionality of Mozzarella cheese. *Australian J. Dairy Technol.* 48(2):99-104.

**Project Title:** Purification of monospecific polyclonal antibodies  
from bovine cheese whey.

**Principal Investigator:** Rodney Brown

**Project Title:** Purification of monospecific polyclonal antibodies from bovine cheese whey

**Personnel:** Rodney J. Brown, Nutrition & Food Sciences Dept., Utah State University  
William H. Scouten, Chemistry & Biochemistry Dept., Utah State University  
Premysl Konecny, Biotechnology Center, Utah State University

**Funding:** Western Center for Dairy Protein Research and Technology

**Objectives:**

The goals of this research are production of monospecific polyclonal antibodies in bovine milk, and purification of these antibodies from cheese whey. Specific research objectives of the project are:

1. Optimization of antibody titers in bovine milk by testing various immunization schemes.
2. Development of a purification method using affinity chromatography with immobilized antigen for purifying antibodies from cheese whey.

**Results:**

a/ Purification of non-specific antibodies by thiophilic chromatography

In the first phase of the project we focused on purification of immunoglobulin G from cheese whey. We presumed that it would be necessary to perform a preliminary purification/concentration of the antibody fraction (mainly immunoglobulin G) from cheese whey prior to obtaining a specific antibody by immunoabsorbent chromatography with an immobilized antigen. This concentration step would allow a higher yield of the antibody of interest. It would also allow us to use the residual immunoglobulin fraction for other purposes after isolating the specific antibody. For example, whey immunoglobulins have been suggested as a prophylactic dietary supplement for newborn calves instead of colostrum, or colostrum-derived preparations enhanced in immunoglobulins.

We have developed a method for the purification of immunoglobulin G (IgG) from sweet cheese whey based on specific interactions with a thiophilic adsorbent. Thiophilic gel (T-gel), a resin of the structure matrix-O-CH<sub>2</sub>CH<sub>2</sub>SO<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>SCH<sub>2</sub>CH<sub>2</sub>OH, has proved to be a useful tool for selective purification of immunoglobulins from various sources, mainly mammalian sera and ascites fluid. The thiophilic interactions are promoted by salt and require presence of lyotropic (water-structure-forming) salts.

Among the salts tested, sodium sulfate most efficiently promoted IgG adsorption on T-gel; 94% of the IgG was bound to the gel at 0.5 M salt concentration, and the amount of IgG eluted reached 75% (Fig. 1). We demonstrated that 0.5 M sodium sulfate was the optimal concentration in thiophilic chromatography of bovine IgG (Fig. 2). The amount of IgG eluted from the T-gel after adsorption of the protein at 40 °C was comparable to that carried out at 20 °C (Fig. 3, Table I). The capacities of two commercially available T-gels for bovine IgG were determined (Table I). The highest eluted (static) capacity achieved was 19.0 mg IgG/ml gel. The purity of IgG prepared by thiophilic chromatography (Fig. 4) was assayed using sodium dodecylsulfate-polyacrylamide gel electrophoresis, radial immunodiffusion and immunoelectrophoresis (Fig. 5, 6).

One-step thiophilic chromatography of crude sweet whey yielded 74% pure immunoglobulin G, and the resulting immunoglobulin retains its antigenic activity, as demonstrated by radial immunodiffusion and immunoelectrophoresis. This method provides very good results compared to the use of highly selective Protein G, which yielded 81% pure immunoglobulin G. For more details see Fig. 1-6 at the end of this report, and the complete data in our article, *J. Chromatogr.*, 673, 45 (1994).

In summary, thiophilic gel, which is more stable, sturdier and less expensive than most of commercially available affinity chromatography materials, proved to be useful for the isolation of bovine immunoglobulin G, yielding protein with a purity comparable to that obtained by Protein G chromatography. The capacities of a randomly chosen commercially available T-gel were found to be reasonable for bovine IgG production. The procedure requires the addition of an appropriate salt (sodium sulfate, potassium sulfate) which can be subsequently removed by ultrafiltration of the IgG-free whey, and recovered from the ultrafiltrate by reverse osmosis and eventually reused or recycled. The volume of cheese whey processed might be reduced by prior ultrafiltration to as little as 10%, which would be a requirement for the use of thiophilic chromatography (batch or column mode) since it would enable one to keep the amount of salt at reasonably low levels. Thiophilic gel can be used at elevated temperatures, so that cooling whey to room temperature would not be required.

#### b/ Antibody production in the milk of dairy cows

Two groups of randomly selected Holstein cows in middle lactation stage (4-5 months after calving) were immunized, each group received a different type of antigen. Dinitrophenol-keyhole limpet hemocyanin (DNP-KLH) and mouse immunoglobulin G (IgG) were chosen as model antigens. DNP is known to be a strong immunogen and keyhole limpet hemocyanin is a preferred carrier due to its phylogenetic diversity from mammal proteins. The DNP-KLH conjugate was expected to be a very good model antigen, stimulating a strong immune response. The rationale

behind the choice of mouse IgG was the potential marketability of the anti-mouse IgG antibody isolated from whey. This antibody might be competitive with anti-mouse IgG antibodies currently being raised in rabbits or goats, having the advantage of being prepared without the necessity of drawing blood from the animals.

Dairy cows were immunized with these antigens as follows. The immunization protocol was submitted and passed a process of peer review and an approval (USU Institutional Animal Care and Use Committee) and was applied as stated.

#### Immunization protocol

animals: dairy cows (Holstein) in early/middle lactation (2 groups of 3, + 2 controls, for a total of 8 cows)

antigens: a. mouse immunoglobulin G

b. dinitrophenol-keyhole limpet hemocyanin conjugate (DNP-KLH)

- concentration: 1 mg/ml, final concentration [after dilution with adjuvant 1:1]

- dose: 10 mg of antigen

administration: subcutaneously, 4 sites (4 x 2.5 ml), cervical region, per immunization period

adjuvant: aluminum hydroxide (Imject Alum, Pierce)

boosters: 2-3 boosters, 3 weeks apart, same dosage as the initial injection

test bleeds: 7-10 ml, from jugular vein

milk collection: weekly

(first test bleeds and milk collection: 7-10 days after the 1<sup>st</sup> booster)

negative controls: serum and milk from the animals on day 0 before immunization, and serum and milk from the nonimmunized cows

Note: a/ The number of animals in each group (3) takes into account the risk of insufficient individual immune response or the loss of an animal, but is not sufficient to provide statistical evaluation of the experimental data.

b/ The choice of adjuvant was limited by the undesirability of using Freund's adjuvant when possible. Application of Freund's adjuvant involves minor stress to the animal, and its use is not desirable with dairy cows. Aluminum hydroxide was chosen as an efficient substitute that provided an enhanced immune response without a stress/danger to animals associated with Freund's adjuvant.

c/ Doses of the immunogens were chosen at an intermediate level capable of producing a good immune response, but are not necessarily optimal. More extensive testing of various amounts of antigens would be necessary for optimization.

### Preparation of whey

Milk was processed into sweet whey as follows. Milk samples (4-6 °C) were centrifuged at 16,000 g for 15 min to separate milk fat. Skimmed milk was warmed in a waterbath to 31-32 °C, and rennet (single strength rennet, diluted 1/20 with water, 4 ml of solution / liter of milk) was added. The milk was then incubated at 31-32 °C for 30-45 min. The resulting gel was cut, and the curd separated from the whey by centrifugation (16,000 g x 15 min). Whey was collected, assayed for the presence of specific antibodies (anti-DNP, anti-mouse IgG), and stored frozen at -80 °C.

### Analysis of antibody titers

We developed an ELISA (Enzyme Linked Immunosorbent Assay) for monitoring titers of specific antibodies against DNP and mouse IgG in both serum and whey. Test bleeds served as necessary controls of the presence of these antibodies. DNP-human serum albumin (DNP-HSA) was used as an antigen in the assay instead of DNP-keyhole limpet hemocyanin due to better solubility of the former. This does not make any difference in assaying the anti-DNP antibody, since it would recognize the DNP group only, regardless of the carrier.

Polystyrene 96-well plates were coated with 100 µl of 0.01 mg/ml DNP-HSA (Calbiochem, LaJolla, CA) or 0.001 mg/ml mouse IgG (Rockland, Gilbertsville, PA) in 0.06 M carbonate-bicarbonate buffer, pH 9.6 by incubation at 4 °C overnight. After washing with phosphate buffered saline (PBS, 0.01M sodium phosphate, 0.15 M sodium chloride, pH 7.20), the plates were incubated with a blocking solution (0.1 g bovine serum albumin/ ml PBS) for 1 hour at 37 °C and washed again with PBS. Sera or whey samples diluted with PBS were added, and then the plates were incubated for 1 hour at 37 °C and washed with PBS. Horseradish peroxidase-anti-bovine IgG conjugate (Sigma, St. Louis, MO) diluted 1/25,000 in PBS was added and incubated for 1 hour at 37 °C. Excess conjugate was washed off with PBS, and the enzyme substrate 3,3',5,5'-tetramethylbenzidine (1-Step Turbo-TMB-ELISA, Pierce, Rockford, IL; 100 µl) was added, followed by 30 min incubation at room temperature. The reaction was stopped with 1 M sulfuric acid (100 µl) and absorbances measured at 410 nm in microplate reader (EL 311, Biotek Instr., Winooski, VT).

Absorbance readings were compared against those of negative controls, i.e. whey and sera samples from non-immunized cows, and whey and sera taken from the cows before immunization. Analysis of antibody titers was done weekly, starting 3rd week after the initial injection.

The ELISA does not allow determination of absolute concentration of anti-DNP or anti-mouse IgG antibodies since there is no standard for these antibodies currently available but it was possible to follow the increase in antibody levels in titers for serum or whey. The difference

between absorbances at 410 nm of the samples and blanks-controls indicating the end-point (end-titer) was set arbitrarily at 0.1 for anti-DNP antibody, and 0.05 for the anti-mouse IgG antibody.

#### Antibody response in serum and whey for DNP-KLH and mouse IgG

Enhanced levels of anti-DNP antibody were detected in blood sera and whey samples from cows No. 7604 and 7802 immunized with DNP-KLH after the second booster injection, i.e. after ca. 40-50 days after the initial injection (Fig. 7). These high antibody titers were observed between days 48-62, and then began decreasing. An increase in anti-DNP titers was observed in serum and whey from cow No. 7604 two weeks after the first booster injection, followed by a slight drop in the titers. Cow No. 7582 gave a very poor immune response throughout.

As expected, anti-DNP antibody titers are significantly lower in whey compared to serum, with a ratio of approximately 1/20-1/100.

One cow (No. 7782) of the group of three injected with mouse IgG gave a good immune response (Fig. 8). Anti-mouse IgG titers peaked two weeks after second and third boosters. There was a good correlation between increase of antibody titers in serum and whey during the immunization period. Anti-mouse IgG titers in whey were 1/10-1/80 lower than those in serum.

Cow No. 7266 died on day 64 from. Autopsy revealed that the cause of death was right site displaced abomasum, and it had no connection with the immunization conducted.

Larger amounts of whey (ca. 6-8 liter each) were prepared from milk from cows No. 7604, 7782, 7802 after antibody titers in serum and whey reached their peaks. The peak titers for these cows were:

cow No. 7604: day 55, anti-DNP titer 1/640,

cow No. 7802: day 55, anti-DNP titer 1/1280; day 62, anti DNP-titer 1/640,

cow No. 7782: day 83, anti-mouse IgG titer 1/320; day 90, anti-mouse IgG titer 1/160.

The whey was prepared as described above and kept frozen at -80 °C for use in immunoaffinity chromatography.

#### c/ Preparation of immunoaffinity adsorbents

We selected two methods of preparation of affinity adsorbents with an immobilized antigen. Among the wide selection of immobilized techniques and commercially available activated gels, tressyl chloride activated agarose and 3M Emphaze represent materials allowing preparation of immunoadsorbents with high binding capacity, low non-specific binding capacities, operating at high flow rates and having potential for regeneration and reuse.

Tresyl chloride (2,2,2-trifluoroethanesulfonyl chloride)-activated gel can be used to couple ligands possessing sulfhydryl and/or amine groups. The activated gel is suitable for immobilizing pH and temperature sensitive proteins, since it is very efficient at neutral pH and even 4 °C.

3M Emphaze Biosupport Medium AB 1 (Pierce, Rockford, IL) possesses an azlactone group, highly reactive with nucleophilic functional groups, typically amines and thiols. Proteins containing primary amines couple to the gel quickly at mild conditions (room temperature, pH 7.5-9.0, 1-2 hours). The character of the support (rigidity, porosity, chemical resistance) allows to use Emphaze in medium pressure, fast flow configurations. It can withstand pressure up to 100 psi and can be exposed to high flow rates (up to 3000 cm/hour) which makes it suitable for large scale applications with high throughputs. Emphaze has been reported by the manufacturer to be easily reused, and derivatized gel retained more than 95 % of its initial capacity over 130 cycles.

Immobilization of proteins of interest (mouse IgG, BSA) on Emphaze was conducted under conditions described. Protein (5 mg/ml in 0.1 M sodium borate buffer / 0.8 M sodium sulfate, pH 9.0) was added to dry gel (5 mg protein / 60 mg dry weight gel, i.e. 10 mg protein / ml wet gel). The coupling reaction proceeded at room temperature for

1 hour, then the gel was thoroughly washed with phosphate buffered saline (PBS; 0.15 M sodium chloride / 0.01 M sodium phosphate buffer, pH 7.20), and remaining unreacted azlactone groups were quenched with 1 M ethanolamine, pH 9.0 at room temperature for

2 hours. After thorough washing with 1.0 M sodium chloride / PBS, pH 7.20, and then PBS, the gel was analyzed for the amount of protein immobilized, using bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) under standard test conditions.

Mouse IgG was immobilized with 85-87% efficiency, resulting in 6.1-6.3 mg mouse IgG bound per 1 ml gel. Immobilization of BSA yielded a derivatized gel with 5.0-5.3 mg BSA / ml gel (63-66 % binding efficiency). The results indicate that using a higher protein/gel ratio in the reaction mixture may yield gels with 15-20 mg protein immobilized per 1 ml gel.

We immobilized BSA on Emphaze and tresyl chloride activated agarose, instead of DNP-HSA. We found out experimentally that the latter had no free amine groups available for binding on the activated gels, as a result of saturation of these groups with DNP. In the alternative approach we will immobilize BSA, HSA or rabbit serum albumin (RSA) on the gel, and then DNP groups will be introduced by reaction with the remaining amine groups of the proteins. It is likely that any of these serum albumins will be a suitable base for DNP groups, since the anti-DNP antibody would recognize only the DNP groups, not reacting with the protein.

#### **Interpretation of the significance of the results:**

a/ We have developed a selective method for purification of IgG from whey. This might be applied as a preliminary purification step prior to isolation of monospecific antibodies using



immunoaffinity chromatography. Alternatively, it might provide, in only one step, fairly pure immunoglobulin G. In either case, it represents a useful tool for antibody purification from whey. It might be employed in antibody production for both veterinary and human needs, and might ultimately lead to increasing the value of cheese whey.

b/ Immunization of dairy cows with two different antigens (dinitrophenol-keyhole limpet hemocyanine and mouse IgG) showed that specific polyclonal antibodies can be produced in milk (whey), which might give us the advantage of preparation of the antibodies without the necessity of drawing blood from the animals.

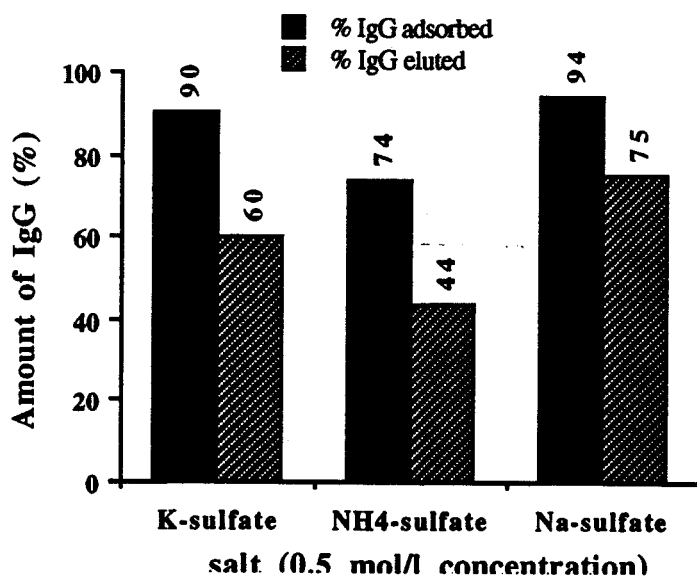
c/ Preparation of immunoabsorbents with immobilized antigens to purify antibodies has been shown to be feasible in preliminary experiments. Further work is required to optimize these procedures.

#### **Future plans:**

In the next part of the project we will focus on developing methods to immobilize DNP and mouse IgG to prepare immunoabsorbents that will be used to purify anti-DNP and anti-mouse IgG antibodies, and we will characterize these immunoabsorbents. We will develop conditions for purification of specific antibodies from whey, and analyze the purity and antigenicity of the final products.

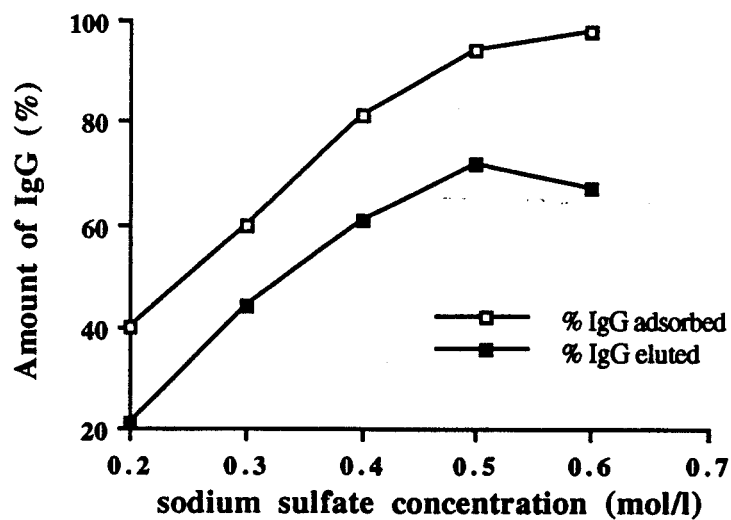
#### **Presentations, publications:**

A paper describing the method for the purification of IgG from whey using the thiophilic adsorbent has been published [P. Konecny, R.J. Brown, W.H. Scouten, Chromatographic Purification of Immunoglobulin G from Bovine Milk Whey, J. Chromatogr., 673, 45 (1994).]. These results were also presented at the 207th American Chemical Society National Meeting, Division of Biochemical Technology session in San Diego, CA in March 1994.



**Fig. 1:** Effect of salts on thiophilic adsorption of bovine IgG

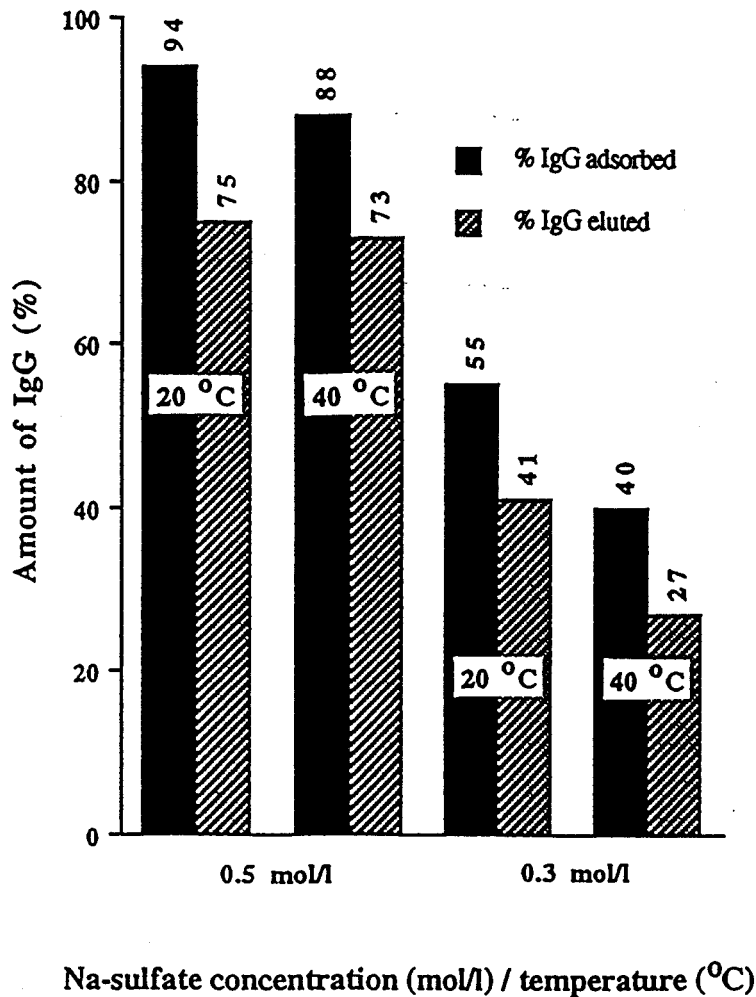
Aliquots (0.5 g) of suction-dried T-gel (matrix: crosslinked 4 % beaded agarose) were equilibrated with 0.5 mol/l salt solutions in 0.05 mol/l sodium-potassium pH 7.5, then treated with 2 ml of IgG solutions (1 mg/ml) for 30 min. The gels were washed with the appropriate salt/buffer, then adsorbed IgG was eluted with 0.05 mol/l sodium-potassium phosphate buffer, pH 7.5. IgG concentration in both washing and elution fractions was quantified. The experiment was done in a batchwise mode. Values reported are the average of five determinations, the standard deviation being less than  $\pm 4\%$ .



**Fig. 2:** Effect of salt concentration on thiophilic adsorption of bovine IgG

T-gel was equilibrated with sodium sulfate solutions of 0.2-0.6 mol/l concentration and then treated as described (Fig. 1). The amount of IgG in washing and elution fractions was determined for various salt concentrations. Values reported are the average of five determinations, the standard deviation being less than  $\pm 4\%$ .

## T-gel: Effect of temperature on adsorption of bovine IgG



**Fig. 3:** Effect of temperature on thiophilic adsorption of bovine IgG

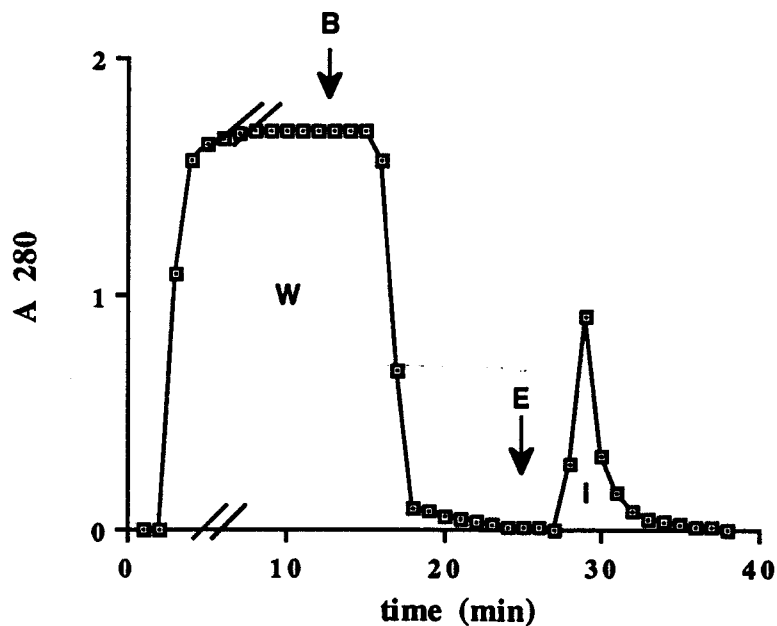
Adsorption of IgG to T-gel (0.5 g aliquots of suction-dried gel) equilibrated with sodium sulfate solutions of 0.3 and 0.5 mol/l concentrations (in 0.05 mol/l sodium-potassium phosphate buffer, pH 7.5) was performed at 20 °C and 40 °C, respectively. Elution was done as described (Fig. 1) at 20 °C and the concentration of IgG in all fractions was quantified spectrophotometrically. Values reported are the average of five determinations, the standard deviation being less than  $\pm 4\%$ .

Table I: Capacity of T-gels and Protein G- Sepharose 4FF for bovine IgG

	Dynamic capacity at 10% breakthrough, at a linear flow-rate of 34 ml/h (mg IgG/ml gel)	Eluted (static) capacity (mg IgG/ml gel)
T-gel (crosslinked 4% beaded agarose)	12.1± 0.3 <sup>a</sup>	12.0±0.4 <sup>a</sup>
T-gel (6% beaded agarose)	18.2±0.3 <sup>a</sup> 17.3±0.5 <sup>b</sup>	19.0±0.4 <sup>a</sup> 18.3±0.5 <sup>b</sup>
Protein G-Sepharose 4FF	22.1±0.4 <sup>a</sup>	19.0±0.6 <sup>a</sup>

<sup>a</sup> at 20 °C

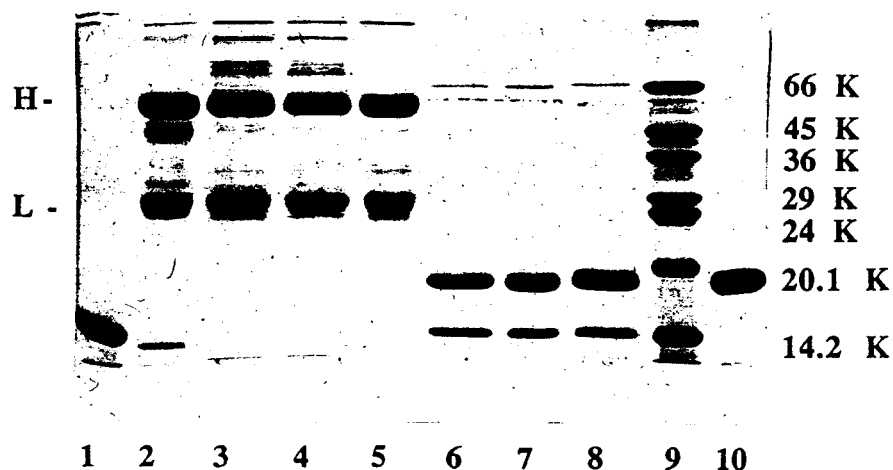
<sup>b</sup> at 40 °C



**Fig. 4:** Purification of IgG from whey by thiophilic chromatography

Clarified sweet whey (85 ml), with solid sodium sulfate added to concentration of 0.5 mol/l, was applied to a column (1.5 cm x 1.3 cm) of T-gel (matrix: 6 % beaded agarose) equilibrated with 0.5 mol/l sodium sulfate / 0.05 mol/l sodium-potassium phosphate, pH 7.5. After washing with the same buffer (B), adsorbed IgG was eluted with 0.05 mol/l sodium-potassium phosphate, pH 7.5 (E). The chromatography was performed at a flow-rate of 1.0 ml/min. Fractions from the chromatography (W: IgG-depleted whey, I: IgG fraction) were characterized by SDS-polyacrylamide electrophoresis (see Fig. 5).

**SDS-PAGE of fractions from chromatographic purifications  
of sweet whey on T-gel and Protein G - Sepharose 4FF**

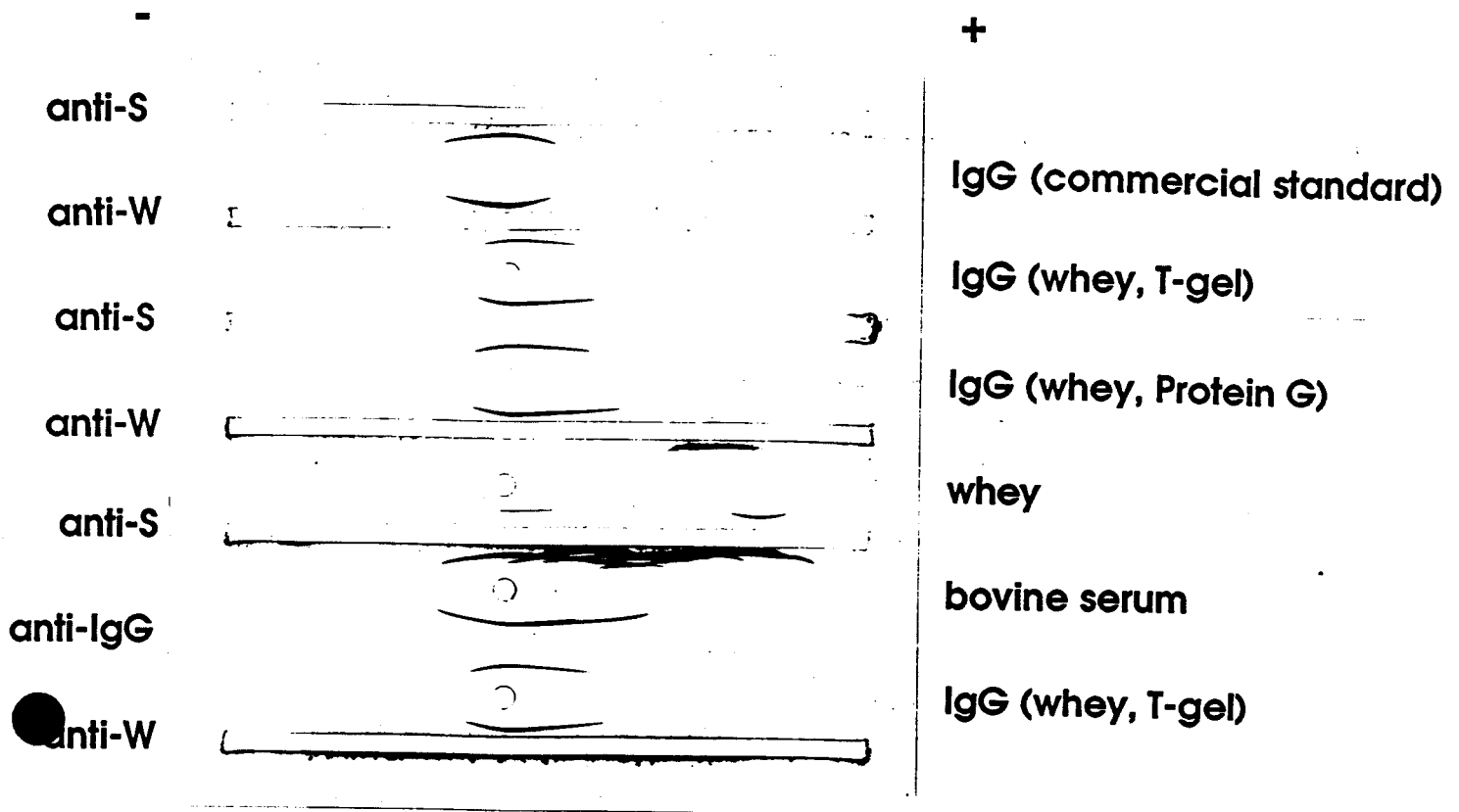


**Fig. 5:** Sodium dodecyl sulfate polyacrylamide electrophoresis (denaturing conditions) of fractions from chromatographic separation of sweet whey on T-gel and Protein G-Sepharose 4FF

SDS-PAGE was run on a 8 cm x 7 cm x 0.75 mm gel (stacking gel: 4 % acrylamide, separating gel: 12 % acrylamide), using the Laemmli buffer system at constant voltage 200 V.

1=  $\alpha$ -lactalbumin, 2=bovine IgG (standard; H= heavy chain, L= light chain), 3= bovine whey IgG from T-gel chromatography at 20 °C (see Fig. 4, fraction I), 4= bovine whey IgG from T-gel chromatography at 40 °C, 5= bovine whey IgG from Protein G-chromatography, 6= IgG-depleted whey after T-gel chromatography (see Fig. 4, fraction W), 7= IgG-depleted whey after Protein G-chromatography, 8= whey, 9= molecular weight standards, 10=  $\beta$ -lactoglobulin

**Immuno-electrophoresis of Bovine Immunoglobulin G purified from whey**



anti-S      anti-bovine whole serum, rabbit

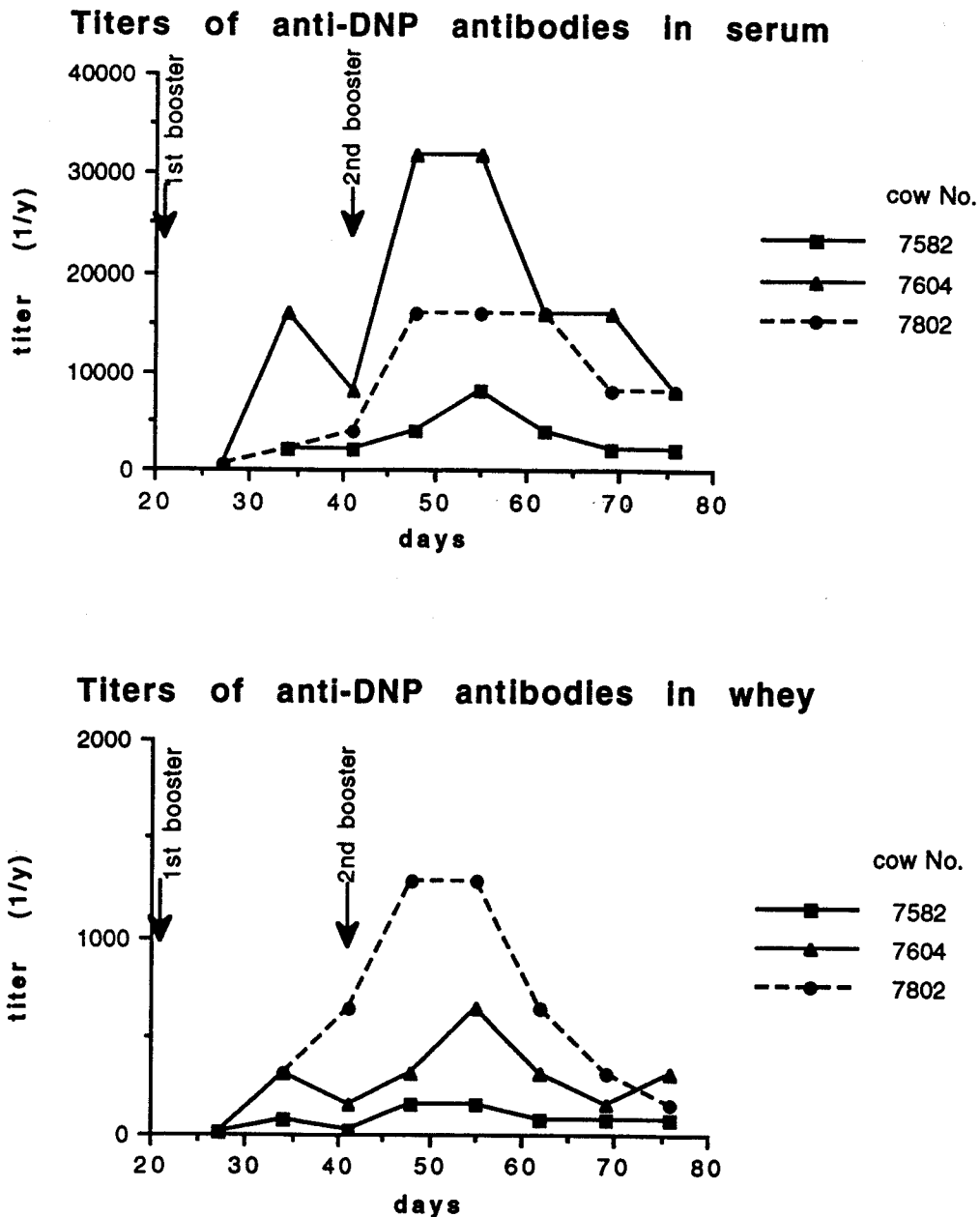
anti-IgG    anti-bovine IgG, rabbit

anti-W      anti-whey proteins, rabbit

**Fig. 6: Immunoelectrophoresis of IgG from whey**

Immunoelectrophoresis was run on 10 cm x 10 cm x 0.1 cm agarose gel (2 % agarose solution in 0.06 M barbital HCl buffer, pH 8.6 / 0.002 % Thimerosal) at constant voltage 60 V (6 V/cm) for 3 hours. Immunodiffusion step (with antisera: anti-bovine whole serum, rabbit; anti-bovine IgG, rabbit; anti-whey proteins, rabbit) was performed at 4 °C for 24 hours, then the gel was dried and stained with Coomassie Brilliant Blue R-250.

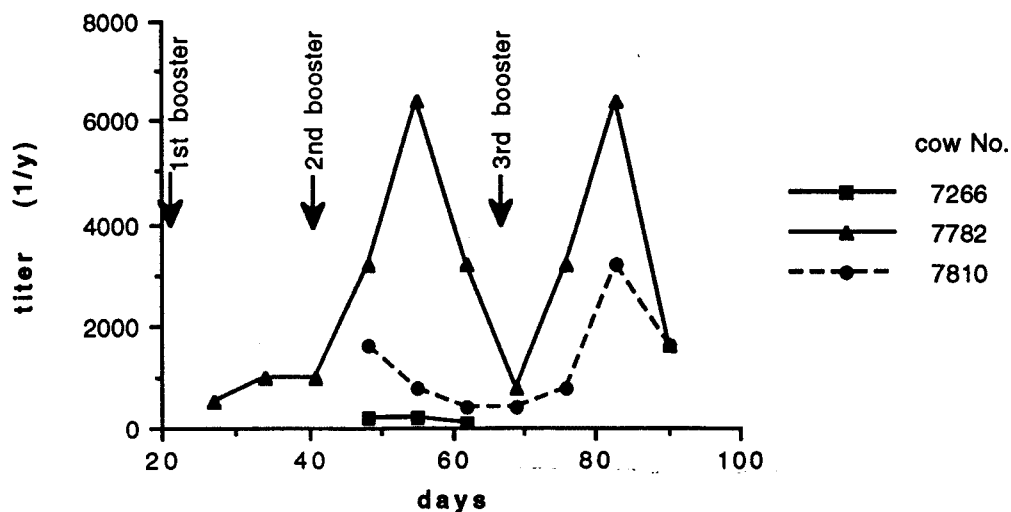




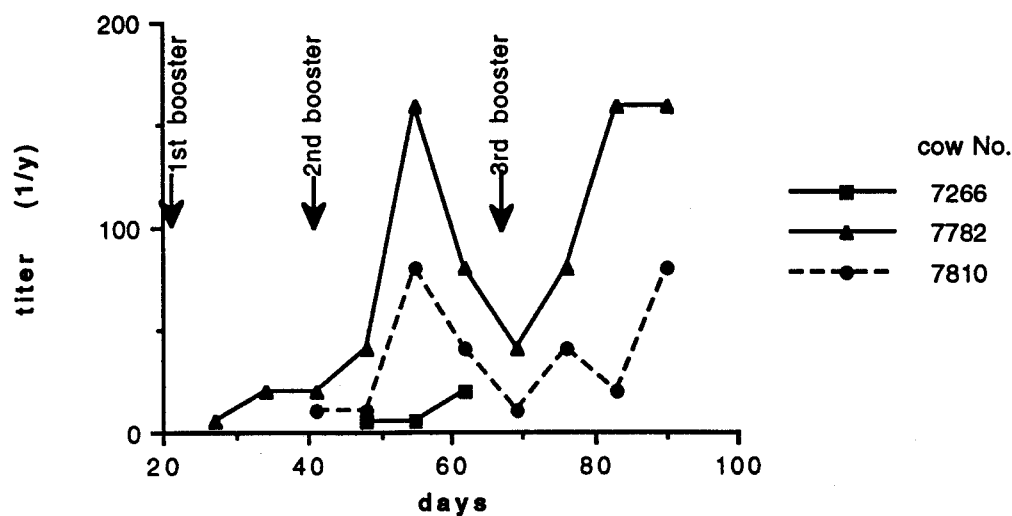
**Fig 7:** Antibody response in sera and whey from cows immunized with DNP-KLH

Cows were immunized with DNP-KLH in combination with Imject Alum (aluminum hydroxide) adjuvant, and boosted on days 21 and 42. Anti-DNP antibody was measured by ELISA. There was no antibody response in sera or whey from control cows (not shown).

### Titers of anti-mouse IgG antibodies in serum



### Titers of anti-mouse IgG antibodies in whey



**Fig 8:** Antibody response in sera and whey from cows immunized with mouse IgG

Cows were immunized with mouse IgG in combination with Inject Alum (aluminum hydroxide) adjuvant, and boosted on days 21, 42, and 66. Anti-DNP antibody was measured by ELISA. There was no antibody response in sera or whey from control cows (not shown).

**Project Title:** Milk protein interactions and gelation during thermal processing.

**Principal Investigator:** Rodney Brown

**Project Title:** Improvement of low-fat cheese through identification isolation and analysis of enzymes and metabolites produced by adjunct cultures during accelerated cheese flavor development (low-fat cheese project).

**Principal Investigator:** Jeff Broadbent

**Project Title:** Improvement of lowfat Cheddar cheese through identification, isolation and analysis of enzymes and metabolites produced by adjunct cultures during accelerated Cheddar cheese flavor development.

**Personnel:** Jeffery R. Broadbent, Dept. Nutrition and Food Sciences, Utah State University.  
Bart C. Weimer, Dept. Nutrition and Food Sciences, Utah State University.  
Mark E. Johnson, Center for Dairy Research, Univ. Wisconsin-Madison.  
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**Funding:** Western Center for Dairy Protein Research and Technology  
Utah Agricultural Experiment Station

### Introduction

Current trends in the American diet clearly indicate that low-fat dairy products will be one of the most important research areas of the 1990's. Unfortunately, traditional cheese flavor is presently not available in reduced-fat varieties, and the inferior flavor and texture of these varieties limits their acceptability among consumers. In general, the lower the fat content, the more difficult it is to produce a cheese similar in quality to full-fat cheese. Starter cultures and media which perform well in the production of full-fat cheese often are not suited to low-fat varieties. As a result, there is a pressing need to develop specialized culture systems that overcome low-fat cheesemaking constraints. Development of new starter systems for low-fat products would be facilitated by more sophisticated knowledge of the role microbial enzymes and metabolites play in cheese flavor development. Studies of cheese maturation have revealed that nonstarter lactobacilli dominate curd microflora during the ripening period and thus are likely to contribute heavily to cheese flavor development. This hypothesis is supported by studies which demonstrated that certain strains of lactobacilli can be used as adjunct starter cultures to improve and accelerate cheese flavor development. Efforts to develop starter systems that improve flavor development in low-fat cheese would be greatly facilitated by a more sophisticated understanding of the role individual microbial enzymes and metabolites play in the maturation process. The most expedient way to approach such a complex problem may be to focus research efforts on cheese flavor adjunct cultures. These bacteria accelerate cheese flavor

development and are used industrially to obtain high quality reduced fat (33% fat reduction) cheese.

This proposal represents a unique collaborative effort between researchers at the Western Center for Milk Proteins Research and Technology at Utah State University, the Center for Dairy Research at the University of Wisconsin-Madison, and members of the Minnesota-South Dakota Dairy Foods Research Center at the University of Minnesota. Together, we seek to systematically identify and characterize key enzymes and metabolites which enable flavor adjunct cultures to accelerate and enhance flavor development in lowfat cheese. Elucidation of key biochemical pathways involved in both desirable and undesirable cheese flavor production would have immediate application in the development of starter systems for manufacture of high-quality low-fat Cheddar cheese.

#### **Objectives:**

1. To systematically characterize enzymes and metabolites implicated in cheese flavor development in starter and flavor adjunct bacteria.
2. To track the microbial conversion of milk proteins into peptides, amino acids and volatile flavor compounds in ripening cheese made with and without culture adjuncts to identify key flavor components which develop more rapidly.
3. To evaluate the role and specificity's of primary proteolysis on cheese flavor development.
4. To characterize the influence of peptidase activity from lactococci, *Lactobacillus casei*, and *Brevibacterium* spp. on cheese flavor development.
5. To characterize the enzymology of amino acid degradation in lactococci, *Lactobacillus casei*, *L. helveticus*, and *Brevibacterium* spp.
6. To characterize a-dicarbonyl production in flavor adjuncts.

#### **Results:**

Research to date has focused on objectives 1-4 as listed above. Forty-eight 550 lb vats of 50% reduced fat Cheddar cheese were manufactured at the University of Wisconsin using a single lactococcal starter or a starter-adjunct pair (Table 1). Starters selected for the study include two commercially used lowfat cultures and one strain known to produce bitter cheese. The adjuncts included four commercial *Lactobacillus* flavor adjuncts and two brevibacteria. In an effort to reduce extraneous influences on flavor development in the cheese, all vats were manufactured under conditions previously shown to minimize contamination by nonstarter lactic acid bacteria. Samples were collected for time 0 analyses, and the remainder was vacuum packaged and stored at 4-7°C for ripening. At monthly intervals, samples of each cheese were removed and analyzed for microbiological, enzymatic and chemical attributes (Table 2). To link changes in cheese composition with flavor properties, expert and consumer taste panels were performed after 2, 4 and 6 mo of ripening. Statistical results from consumer taste panels have

demonstrated that both starter and adjunct made significant contributions to flavor development. Efforts are now underway to draw correlations between taste panel conclusions and the chemical, enzymatic and microbiological data we have collected on each cheese over time.

TABLE 1. Starter-adjunct combinations used in the study<sup>1</sup>.

Starter	<i>Lactobacillus casei</i>		<i>L. helveticus</i>		<i>Brevibacterium linens</i>		
MM100	LC201	LC202	LH32	LH212	BL1	BL2	Control <sup>2</sup>
MM11	LC201	LC202	LH32	LH212	BL1	BL2	Control <sup>2</sup>
MM210	LC201	LC202	LH32	LH212	BL1	BL2	Control <sup>2</sup>

<sup>1</sup>All combinations were manufactured in duplicate.

<sup>2</sup>Duplicate starter-only control vats were prepared at the beginning and end of the make schedule.

TABLE 2. Characterization of cheese flavor attributes in 550 lb. vats of lowfat Cheddar cheese.

<u>USU</u>	<u>UW</u>	<u>UM</u>
Sensory analysis and statistics (consumer preference panels)	Sensory analysis and statistics (trained panels)	Free fatty acids (GC)
Free amino acid content (CE, amino acid analysis)	Non starter bacteria levels (microbiological)	Ammonia content (GC)
Peptide content (CE)	Starter levels (microbiological)	H <sub>2</sub> S content (GC)
<i>p</i> -cresol, indole and skatole (CE)	Adjunct levels (microbiological)	Methanethiol content (GC)
Aminopeptidase activity (colorimetric)	Redox potential (potentiometer)	Levels of other sulfur-containing volatiles (GC)
X-PDAP activity (colorimetric)	Methylglyoxal, glyoxal & diacetyl (HPLC)	
	D/L lactic acid (commercial kit)	

### Significance to the Dairy Industry

Future acceptance and demand for low-fat cheese will be heavily dependent on the availability of high-quality products. Solutions to the flavor and textural problems that have dogged low-fat cheese manufacture will require a more comprehensive understanding of the role other microbial enzymes and metabolites play in cheese flavor development. This proposal seeks

to identify and characterize specific enzymes and metabolites which enable cheese flavor adjunct cultures to accelerate cheese flavor development. Identification and characterization of these properties will facilitate the development of low-fat starter systems, through strain combinations or recombinant DNA technology, for the manufacture of high-quality low-fat cheese. Low-fat cheese with organoleptic qualities of full-fat varieties will increase consumer acceptance of low-fat dairy products and expand consumer demand for these goods to individuals that avoid cheese for reasons of diet and the absence of high quality low-fat alternatives.

**Publications**

none in 1993-94

**Abstracts**

Strickland, M. and B.C. Weimer. 1994. Use of capillary electrophoresis for detection of phenylalanine, tyrosine, tryptophan, indole, skatole, p-cresol, caseins and peptides in Cheddar cheese. *J. Dairy Sci.*(Suppl. 1) 77:



**Project Title:** Selection of lactococcal starter strains for use in low-fat cheese manufactured based on their hydrolysis of milk proteins (low-fat cheese project).

**Principal Investigator:** Bart Weimer

**Project Title:** Selection of lactococcal starter strains for use in low fat cheese manufacture based on their hydrolysis of milk proteins.

**Personnel:** Bart Weimer, Dept. of Nutrition and Food Sciences, Utah State University  
Bart DeVries, Dept. of Nutrition and Food Sciences, Utah State University

### Introduction

Future acceptance and increasing demand for low fat cheese will be heavily dependent on the availability of starter cultures that consistently produce a high-quality, flavorful product. Development of these cultures will in turn be dependent upon identification of key metabolic functions, which promote desirable cheese flavor, texture, and body, and their relationship to bacteriophage (phage) resistance. Production of full fat cheese is still plagued with problems associated with bacteriophage during cheese making and low fat cheese makers will be faced with fewer starters to choose from that will make an acceptable product. This dichotomy between phage resistance and starters with desirable production characteristics has left the cheese maker with a hard full of strains for manufacture of low fat cheese.

Low fat cheese manufacture presents unique demands for lactococcal starter cultures in terms of flavor production and phage resistance. Traditional strains, commercially available, have proven to produce inferior low fat cheese even though they produce high quality full fat cheese. One important difference for low fat cheese starters is that the strains used must produce acid at a slow rate, so acid development can be controlled during cheese making.

Derivation, widely used in New Zealand and Australia to obtain phage resistant variants, yields many slow acid-producing mutants that have been discarded in the past. However, phage derivation also changes many biochemical characteristics that influence proteolysis and subsequent flavor development. These changes also alter optimum growth parameters for bulk starter production when used for cheese making. To consistently produce a high quality, low fat cheese commercially, it is necessary to isolate and characterize starter cultures specifically for use in low fat cheese. It is important to develop a bank of cultures suitable for use by commercial cheese manufacturers that will be phage resistant.

In Australia, derivation has been used successfully in factories to produce many phage resistant starters used industrially for many years. This technique has provided a continuing supply of uncharacterized starters that have varying genetic and metabolic characteristics as compared to the parent. A need exists to have a bank of phage resistant starters for low fat cheese production that are characterized with regard to proteinase, peptidase, lipase, and other key enzymes that contribute to cheese flavor, texture and body of cheese.

### Objectives

- A. Select slow acid-producing starter strains (both *Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris*) that have proven quality records in full fat cheese manufacture. Isolates will be compared to benchmark strains that are currently used for low fat cheese production
- B. Examine effect of media and environmental parameters on growth, proteinase, and peptidase activity.

- C. Characterize effects of derivation for phage resistance on the phenotypic expression of  $\beta$ -galactosidase, proteinase, peptidase, and metabolism of parents and phage-resistant derivatives.

### Results

Slow acid-producing phage resistant starter strains have been isolated from three strains of lactococci used to make good quality low-fat and high fat Cheddar cheese. These phage-resistant strains have been partially characterized for phenotypic changes due to selection (Table 1). Changes are mutant dependent and are found in enzyme systems associated with cheese flavor which influence the commercial utility for flavor development and acid production in cheese production. Characterization of some traits are still underway for the mutants. Initial results indicate  $\beta$ -galactosidase, proteinase, peptidase, and metabolism of the mutants are significantly different from the parent strain.

Early results from objective 2, determination of the influence of media composition, indicate media has a significant effect on the peptidolytic and lipolytic activity of the strains tested. This is an important finding and suggests bulk media composition will impact starter culture performance and flavor development via enzyme system activation during bulk starter growth. These data provide insight into which enzymes can be manipulated with media composition and what impact they will have on cheese flavor when bulk starter cultures are used in cheese manufacture.

Table 1. Characteristics that will be determined for parents and derivatives.

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Acid production (coloimetric assay and pH [Pearce, 1969])
General metabolic characteristics - 95 biochemical attributes (Biolog)
Salt tolerance - 0 to 5% (coloimetric assay)
Arginine metabolism (Differential broth)
Phage host range (coloimetric assay, plate assay)
General proteinase activity (chromogenic assay, capillary electrophoresis)
Total aminopeptidase activity (chromogenic assay)
Lipase/esterase activity (chromogenic assay)
Genetic characterization (Pulse field Gel Electrophoresis, plasmid profile)
$\alpha$ -dicarbonyl (King Test [Vasavada and White], HPLC, capillary electrophoresis)
<i>p</i> -cresol, indole, and skatole (HPLC, capillary electrophoresis)
Glutathione - cellular content (HPLC [Fernandez and Steele, 1993])

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### Significance to the Dairy Industry

Phenotypic changes due to selection of phage resistant mutants significantly impact the flavor of cheese. This work highlights the importance of the culture characterization and

suggests metabolic traits that influence cheese flavor can be controlled and may need to be accounted for when cultures are rotated for phage resistance. Determination of the growth conditions which influence the enzyme systems that contribute to desirable cheese flavor will lead to recommendations to the cheese maker that will minimize undesirable flavor systems while maximizing desirable characteristics.

**Publications**

none in 1993-1994

**Abstracts**

none in 1993-1994

**Project Title:** Time and processing effects on water interaction with the protein-reduced fat matrix (low-fat cheese project).

**Principal Investigator:** Conly Hansen

July 28, 1994

**Time and processing effects on the water interaction with the protein-reduced fat matrix**

Report to the western dairy foods research center

Antonio Torres and Conly L Hansen, Co-Principal Investigators

**INTRODUCTION**

Manufacture of high quality low-fat cheese (with  $\geq 50\%$  reduction in fat compared to its full-fat counterpart) has proven difficult because these types of products typically lack characteristic flavor and texture attributes of full fat cheeses.

When a significant percentage of fat is removed from milk for the manufacture of low-fat cheeses, the rheological properties and texture of the cheeses are adversely affected. A common defect is in the cheese texture. Removal of fat from milk increases the protein content. It is thought this makes the texture of cheese too hard and plays a role in the lack of flavor development in low-fat cheeses. There are many factors that affect the quality of low-fat cheese. One factor that must be explored is to determine how manufacturing conditions affect microbial growth and protein functionality and breakdown in low-fat cheeses. The principal investigators listed above are part of the team researching how to improve the quality of low-fat cheese.

## Research Objective 3

Determine how manufacturing conditions affect microbial growth and protein functionality and breakdown in low fat cheeses.

### Research Strategy 3.1

Fat reduction effect on the water interaction with the protein-fat matrix.

#### Specific Objectives:

- A. Prepare cheese at minimum, 75% reduced, 50% reduced, 33% reduced and full fat level following curd handling procedures used in industry. (Year 1 and 2).
- B. Determine during early storage (under 30 days) and at constant temperature the changes of the state of water in the reduced fat - protein matrix. Measurements used include differential scanning calorimetry (DSC) and moisture. (Year 1 and 2).
- C. Determine during early storage (under 30 days) the state of water in cheese slabs as affected by temperature gradient and location in the slab. (Year 3)
- D. Determine during early storage (under 30 days) structural changes with time and temperature of the cheese matrix by electron microscopy. (Year 1 and 2)
- E. Develop and validate a moisture transfer model by determining the driving forces affecting free whey generation and its movement in the cheese matrix and quantifying the appropriate model parameters. (Year 2 and 3).
- F. Make process recommendations for the handling of low fat cheese curd and pressing into blocks. (Year 3).

#### KEY DELIVERABLES (first year):

1. Cheese microstructure changes in a few days from a porous sponge-like material with interconnected channels (diameter  $\approx 10 \mu\text{m}$ ) in freshly pressed cheese to a continuous protein matrix with embedded fat particles. This provides a new interpretation for the locking of moisture variability in Cheddar cheese blocks and should lead to processing conditions reducing this variability.
2. The presence of interconnected channels during the time that moisture gradients are established in a cheese block suggest that moisture movement within cheese blocks may be described as whey flow in a porous media.
3. Temperature differences affect rate of free whey production but do not explain its movement within a cheese block. This suggests that moisture gradients could be reduced even if temperature gradients are not eliminated.

**WORK DONE:** Experimental work completed (first year):

1. Cheese has been prepared at three different fat levels:
  - minimum fat
  - 50% reduced
  - full fat cheese
  
2. Curd treatments included so far have been:
  - A - stirred curd
  - B - curd and whey mixture cooled to 70° F (\*)
  - C - curd and whey mixture cooled to 80° F (\*)
  - D - curd washed with cold water to 70° F
  - E - curd washed with cold water to 80° F
  - F - curd and whey mixture cooled to 70° F (\*) and minimum water rinse
  - G - curd and whey mixture cooled to 80° F (\*) and minimum water rinse
  - H - stirred curd (vat A replicate for additional variability assessment)(\*) chilled water through the jacketed wall
  
3. Sample handling procedures were as follows. Each vat yielded one cheese block ( $\approx$  20 lb) which was cut into small size chunks (2"x1.5"x5") for faster equilibration to storage temperature (under 2 hours).
  - Storage temperature: 4° C, 13° C, and 25° C.
  - Storage time: 1, 2, 5, 10 and 21 days.
  - Measurements included DSC analysis, moisture content and pH.

## EXPERIMENTAL RESULTS

The data obtained and analyzed so far indicates that the volume of whey expelled from cheese is affected by temperature and curd handling procedures. It is important to note that such expulsion occurs even in the absence of temperature differences within the cheese samples. For example, Figure 1 (minimum fat cheese, i.e.,  $\approx$  1% fat) shows a large drop in moisture content in samples stored at 25° C even though the initial sample temperature is approximately the same as the storage temperature. Furthermore, the small size chunks (2"x1.5"x5") used in our experiments can reach 4° C and 13° C in  $\approx$  2 hours. Therefore, whey movement to the block surface occurs even in the absence of temperature gradients. The driving force for this whey movement will be determined in years 2 and 3 of this project.

A related experiment was the preparation of thin curd layers (Figure 2) stored at constant temperature for approximately 24 hours. These thin layers were pressed at each sampling time and the amount of whey expelled was estimated by recording changes in the weight of the pressed curd. The curves obtained suggest a first order kinetics behavior in the 5-30° C temperature range. A comparison between Figures 1 and 2 shows that in the small size chunk experiments no significant differences are observed between 4° C and 13° C. This observation might reflect the relatively large distance that the whey needs to travel to reach the surface in the case of the cheese chunks as compared to thin curd layers. Also, in the latter experiment the samples were pressed at each sampling time. This preliminary experiment suggests a mean to differentiate between free



whey generation and free whey movement. Combining this information with pH changes (Figure 3) we will propose a mechanistic model for moisture variability in natural cheese blocks.

A mechanistic interpretation of moisture movement in natural cheese requires information on the moisture matrix interaction. Differential scanning calorimetry (DSC) can provide such information. A DSC unit donated by the Technology Center at Kraft General Foods to support this project is being used to quantify the energy involved in phase transitions as a function of storage time and temperature, fat level, and curd handling (Figures 4 and 5). The ice melting peak area remains more or less constant after an initial drop (Figure 4). Note that peak area, expressed as energy (J) per sample size (gram of cheese) depends on the moisture content of the sample. Therefore, a better parameter is the amount of energy involved per gram of water divided by the amount of energy per gram of pure water. This parameter ("free water index", FWI) reflects the water fraction participating in the phase transition. Figure 5 shows changes in FWI occurring early during storage time. We are currently determining whether FWI is affected by curd handling technologies.

Electron microscope (SEM) observations show cheese microstructure changing in a few days from a porous sponge-like material with interconnected channels (diameter  $\approx 10 \mu\text{m}$ ) in freshly pressed cheese to a continuous matrix with embedded fat particles. This provides a new interpretation for the locking of moisture variability in Cheddar cheese blocks. Whey movement is reduced to near zero when the interconnected channels disappear.

In summary, the first phase of this study shows that factors involved in moisture migration in Cheddar cheese block are active during storage (probably less than 5 days). Moisture movement in chunks, syneresis in thin curd layer, DSC determinations, and SEM structure changes were observed only during early sample storage.

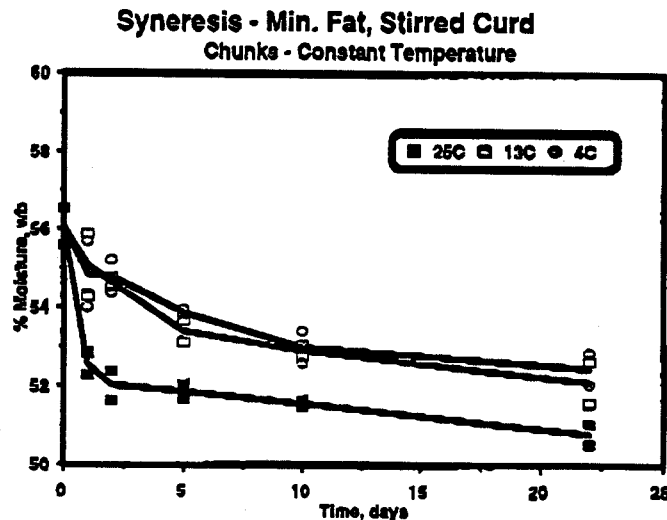


Figure 1

**Syneresis - 33% Reduced, Stirred Curd**  
Thin Layer - Constant Temperature

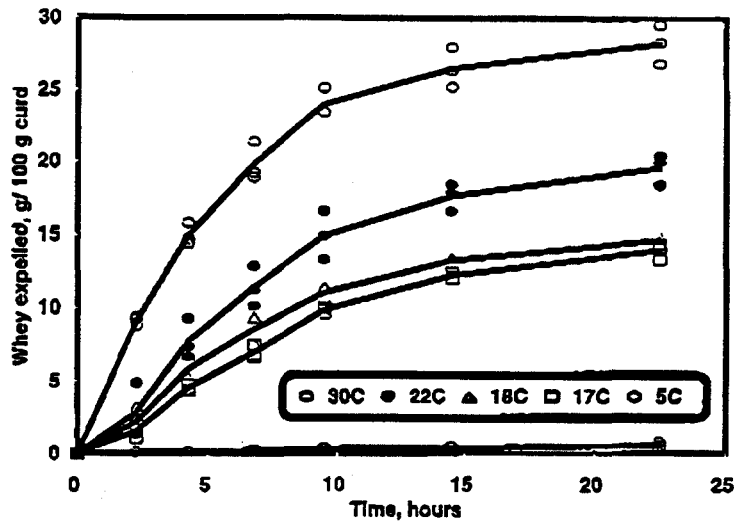


Figure 2

**pH - Min. Fat, Stirred Curd**  
Chunks - Constant Temperature

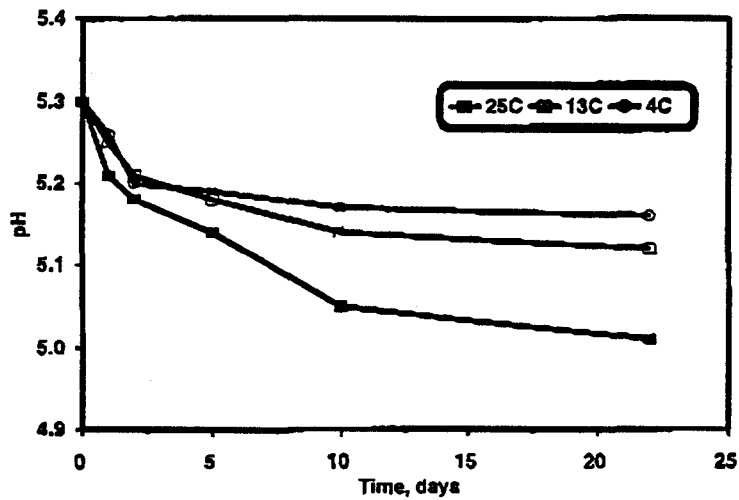


Figure 3

**DSC peak - Min. Fat, Stirred Curd**  
**Chunks - Constant Temperature**

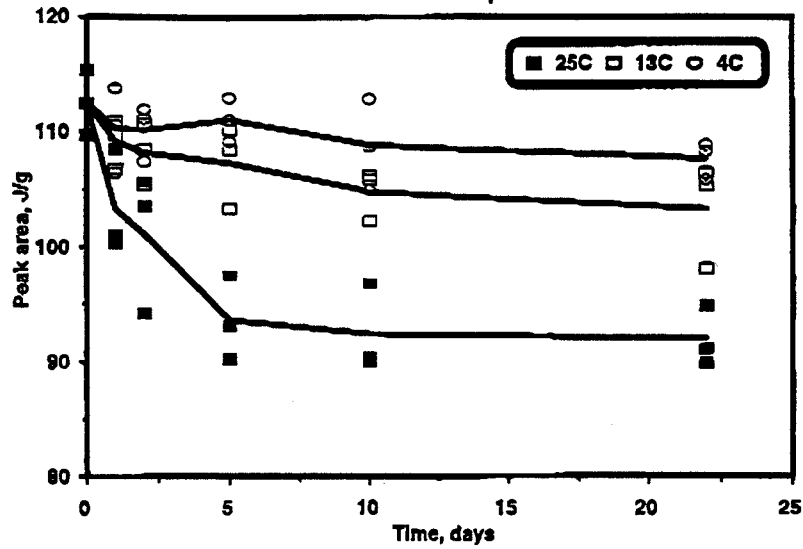


Figure 4

**FWI - Min. Fat, Stirred Curd**  
**Chunks - Constant Temperature**

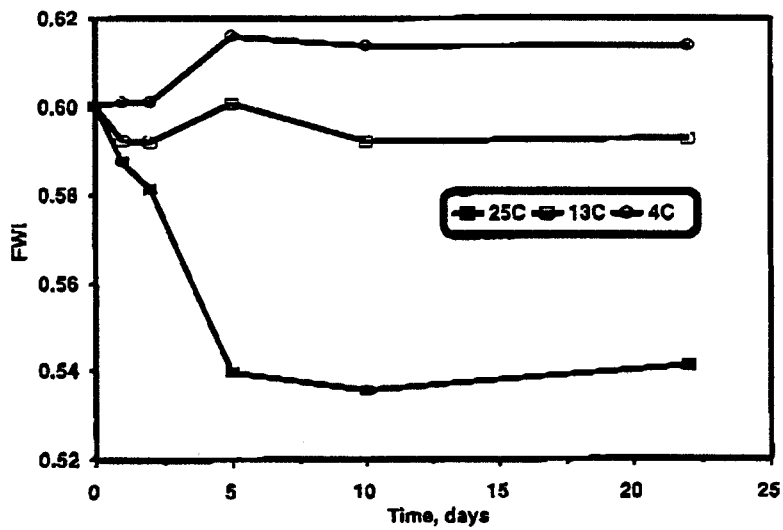


Figure 5

**Project Title:** Time and processing effects on water interaction with the protein-reduced fat matrix (low-fat cheese project).

**Principal Investigator:** Antonio Torres

**Project Title:** Proteinase activities from new strains of  
*Lactococcus lactis* subsp. *cremoris* (low-fat cheese  
project).

**Principal Investigator:** Bruce Geller

**Title:** Proteinase activities from new strains of *Lactococcus lactis* subsp. *cremoris*

**Personnel:** Bruce Geller  
Department of Microbiology  
Oregon State University

**Funding:** Western Center for Dairy Protein Research and Technology

#### Introduction

The objective of the project is to characterize and evaluate recently isolated, unique strains of *Lactococcus lactis* subsp. *cremoris* for proteolytic specificities, and for their potential use in low fat cheeses. The characterization focuses on proteolytic activity because it is necessary for the acid production required in milk fermentations, and is a determinant of the flavor in cheeses. Ultimately, our goals are to identify the characteristics among these new strains of *L. lactis* subsp. *cremoris* that are favorable for making low fat cheeses, and to understand the chemical and biological differences between strains that account for the differences in their abilities to make high quality cheeses.

Nearly all commercial and laboratory strains of *L. lactis* subsp. *cremoris* are derivatives of just a few unique strains. Many attempts to isolate different strains have failed, leaving the fermented dairy foods industry with a limited variety of genetic and biochemical characteristics in this subspecies.

Recently, two labs in our department have collaborated on a NDPRB-funded project, and isolated 28 new strains of *L. lactis* subsp. *cremoris*. Five of these strains have been tested by seven independent methods, and are genetically and biochemically different from one another, as well as from any of the known *cremoris* strains. Our lab is characterizing these strains in the following way: 1) determining the specificities of the proteinases by the electrophoretic pattern of the products of casein hydrolysis, 2) making and testing a low fat cheddar cheese and 3) cloning and sequencing the gene(s) for proteinases with unique proteolytic specificities.

#### Specific Objectives

In broad terms, the objective of the project is to characterize and evaluate 28 recently isolated and unique strains of *Lactococcus lactis* subsp. *cremoris* for proteolytic activities, and for potential use in low fat cheeses.

To accomplish this, the project has the following specific objectives:

- a) Determine the specificities of the proteinases by analyzing the cleavage products of the casein substrate.
- b) Make and analyze a low fat cheddar cheese using one or more of the new strains.
- c) Clone and sequence the gene(s) for proteinases with unique proteolytic specificities and favorable cheese-making characteristics.

#### Significance

Our project is to characterize newly isolated strains of *Lactococcus lactis*

subsp. *cremoris*, starting with the one of the most important characteristics of the starter culture, the proteinase activity. There are many significant reasons why the proposed research will benefit the dairy industry. First, low fat cheese is now made from only a few acceptable strains of subsp. *cremoris*, and the proposed project would identify those newly isolated, unique *cremoris* strains with the proteinase characteristics required for commercial use. Second, our approach to the problem of a lack of commercially usable *cremoris* strains takes advantage of the natural pool of *cremoris* traits that have gone untapped. Much emphasis has been placed recently on modifying existing *cremoris* strains using genetic engineering, particularly by the European dairy research groups. However, our proposal would avoid the legal and commercial uncertainty of using genetic engineering, and characterize new, potentially usable, and naturally-isolated strains of subsp. *cremoris*. Third, preliminary tests have shown that the new strains have characteristics which are different from existing strains of *cremoris*, and which are favorable for cheese-making (such as fast acid production and phage-resistance). This indicates that the new strains are potentially usable for making cheese, and have characteristics previously unknown in other *cremoris* strains. Fourth, our approach is to understand a chemical/enzymatic basis for differences between the strains' abilities to make a better cheese. This would give the industry a rational way of screening new strains in the laboratory, without having to test each strain by trial and error in a cheese vat. Fifth, by isolating the proteinase genes from these "new" strains, they could be transferred to other strains to produce unique combinations of characteristics important for cheese-making.

#### Results

Twenty-eight of the new *cremoris* isolates have been screened for their proteolytic degradation patterns of casein. Two appear to have unique patterns that are different than any of the three known patterns. Thus, at least two of the new strains appear to be different in their proteolytic degradation of casein than any of the existing *cremoris* strains. We are currently screening more of the strains for their casein degradation patterns, including two that taste-tested bitter. We have also started a more detailed characterization of the proteinases with unique patterns, which includes a kinetic analysis of the crude proteinase preparations, and an analysis of the degradation of the individual casein subunits. In collaboration with the Sandine and Giovannoni labs and Tillamook Dairy, we are planning to make and test a low fat cheddar cheese using at least one of the strains that we have identified as having unique proteolytic activity.

#### Deliverables

For FY95 (07/01/94-06/30/95), the deliverables are:

1. Identify strains of *Lactococcus lactis* subsp. *cremoris* with unique patterns of casein degradation.
2. Make a low fat cheese with at least one of the new *cremoris* strains, and analyze it by objective and subjective criteria.
3. Identify and characterize the peptides formed from the hydrolysis of casein by the unique proteinases of the new *cremoris* strains.

**Project Title:** Preliminary examination of hazelnut enzymes extracts for flavor enhancement of reduced fat Cheddar cheese (low-fat cheese project).

**Principal Investigator:** Floyd Bodyfelt



## PROGRESS REPORT

**Project Title:** Preliminary Examination of Hazelnut Enzyme Extracts for Flavor Enhancement of Reduced Fat Cheddar Cheese

**Personnel:** Floyd W. Bodyfelt  
Michael H. Penner  
Dan Smith  
Dept. of Food Science and Technology, Oregon State University

**Funding:** Western Center for Dairy Proteins Research and Technology

### Introduction:

In previous work at the OSU Department of Food Science and Technology (~1950), a crude enzyme extract of hazelnut enzymes (HEE) was applied to Cheddar cheese curd for the purpose of developing aged cheese flavor within 1-2 weeks time. This study was terminated primarily because the protease and/or lipase activity could not be controlled; developed flavors became too intense. Recent application of high hydrostatic pressure (2000-4000 atmospheres) treatment has demonstrated the potential for inactivation or control of enzyme systems without destroying sensory properties of cheese. Thus, this study is a preliminary examination of the feasibility of developing more appealing flavor and textural properties of HEE treated lowfat Cheddar cheese.

### Objectives:

1. Assay the protease and lipase activity and ascertain the extent of potential bitterness developed in 2% UHT milk with added hazelnut enzyme extracts.
2. Determine the optimum high pressure treatment for HEE inactivation.
3. Determine and describe the effects of high pressure treatment on the sensory and rheological properties of Cheddar cheese.
4. Characterize the proteolytic, lipolytic and flavor imparting properties of various hazelnut preparations.
5. Evaluate the suitability of propylene oxide sterilized hazelnuts as a source of HEE and "whole nut" preparation(s).
6. Study and refine methodologies for preparation and incorporation of potential flavor imparting hazelnut preparations onto cheese curd.
7. Conduct necessary cheesemaking trials to optimize incorporation of HEE and whole hazelnut preparation; ascertain proteolysis, lipolysis and sensory properties modification of lowfat cheese.

### Results:

Hazelnut meats were obtained from an Oregon processor (Westnut, Inc., Dundee). Hazelnut enzyme extracts (HEE) were prepared using (1) 1.0 M NaCl and (2) 0.05 M phosphate buffer (pH 7.0) with subsequent purification by salting out with ammonium sulfate. The aqueous portion of the extracts was obtained by centrifugation and filter sterilized (0.2  $\mu$ m). Cartons of 2% UHT milk (236 ml) were aseptically inoculated in duplicate with aliquots of each extract (4% by volume), incubated for 3 and 6 days at 7.2°C, and then examined for the appearance of proteolytic products using the OPA method of Church, *et al.* Both extracts produced significant amounts of proteolysis in the milk within three days. The detectable proteolytic products in milk treated with the NaCl extract were 3-4 times greater than the assay's background detection of amino acids and

peptides in untreated milk. Detectable proteolytic products in the phosphate buffer extract treated milk were 2-3 times above background. The phosphate buffer extract showed much better stability, as the NaCl extract lost almost half of its proteolytic activity when held at 4°C for 24 hours prior to inoculating the milk. Beyond day 3 of incubation the proteolytic activity in both extracts diminished markedly. Treatment of inoculated milk samples with high pressure (3500 atm for 30 min) terminated proteolytic activity.

A trained sensory panel did not detect bitterness or other off-flavors in sterile filtered HEE treated milk samples. Total plate counts showed them to be free of microbial contamination.

Lipase activity was estimated by titration of free fatty acids produced in HEE inoculated 2% UHT milk. Negligible amounts of fatty acids were detected, perhaps owing to the low milk fat. The NaCl hazelnut extract exhibited esterase activity toward artificial substrates of chain lengths C4 to C16. The phosphate extract is still to be tested for lipase/esterase activity.

Low fat cheddar curd was treated with 2% (volume of NaCl extract/weight of curd) HEE. To date, at ten weeks aging at 7.2°C, the sensory panel has not observed any difference in flavor characteristics between the HEE treated and control low fat cheddar. There is no significant difference in proteolytic products as measured by the OPA assay.

Microbial contamination of the hazelnuts and instability of the proteolytic activity in the HEE are two challenges that have been partially overcome. The microflora present on the hazelnut surface confounded the initial observations in milk. Hazelnuts treated with propylene oxide gas, provided by Westnut, have a much lower plate count while retaining most of the proteolytic activity. The use of nisin or irradiation of the HEE are also under consideration as controls of incidental contamination. Ideally, we hope to provide an extraction protocol that avoids the need for a somewhat cumbersome sterilizing filtration. As noted above, HEE is more stable in phosphate buffer than the NaCl solution. We are working to optimize both initial activity and stability of the extract. Freeze drying has also shown promise for the retention of enzyme activity in this extract.

#### **Significance to the Industry:**

Recent consumer trends toward consumption of lower fat content foods prompts the dairy industry to closely examine all technological approaches toward the development and production of higher quality, more appealing lowfat Cheddar cheese. This study represents another possible means of attaining this industry goal.

#### **Deliverables FY 95:**

It is our intent to determine an optimum method(s) to separate and utilize either hazelnut enzyme extracts and/or incorporate a whole hazelnut preparation (i.e. semi-flowable powder) for the potential treatment of lowfat Cheddar cheese curd for enhancement of lowfat cheese flavor properties.

**Project Title:** Development of thermophilic cultures for  
manufacture of low-fat and non-fat Mozzarella  
cheese (low-fat cheese project).

**Principal Investigator:** Donald McMahon

**Project Title:** Development of Thermophilic Cultures for  
Manufacture of low-fat and non-fat Mozzarella  
Cheese.

**Personnel:** Donald J. McMahon  
Craig J. Oberg

**Funding:** WESTERN CENTER FOR DAIRY PROTEIN  
RESEARCH AND TECHNOLOGY  
Utah Agricultural Experiment Station

**Objectives:**

In the manufacture of low fat and non fat Mozzarella cheeses it has been well recognized that the moisture content of the cheese must be increased without having "weeping" of whey from the cheese after packaging. It has also been shown that low fat Mozzarella cheese takes a longer time to "mellow" and develop good melting properties. This process could perhaps be accelerated by increasing the proteolysis occurring in the cheese during storage. Both of these factors (increasing moisture and increasing proteolysis) can be manipulated through use of appropriate starter cultures.

- A. Develop thermophilic starter cultures, including *Streptococcus thermophilus*, *Lactobacillus helveticus*, *Lactobacillus delbruekii* ssp. *bulgaricus*, and *Lactobacillus casei* strains that will be suitable for use in the manufacture of low fat and non fat Mozzarella cheese.
- B. Manufacture low fat and non fat Mozzarella cheese using cultures developed in objective A that have the desirable physical properties of part-skim Mozzarella cheese.

**Results:**

Some cultures that have been reported to increase moisture content of Mozzarella cheese have been obtained. Also some Lac<sup>-</sup> Prt<sup>+</sup> cultures have been obtained. The graduate students who are to work on this project did not commence their program until June 1994. Preliminary cheesemaking trials have been conducted but no results are available at this time.

**Publications:**

Nil

## **Operational Advisory Committee Business Meeting Agenda**

1. Acceptance of 1993 OAC Meeting Minutes
2. WCDPRT Financial Report
3. Review of 1994 Technical Advisory Committee Report
4. Approval of research for FY95
5. Committee Reports
  - Structure of Center - Chairman: Donald McMahon
  - Research directions - Chairman: Floyd Bodyfelt
  - Industry support - Chairman: Carl Brothersen
  - Role of the Center in the West - Chairman: Mark Daeschel
6. Direction of the Center for the next contract period