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WESTERN DAIRY CENTER  
for Dairy Protein Research  
and Technology

ANNUAL REPORT  
1992 - 1993

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## WDFRC ACTIVITIES SUMMARY

The Western Center for Dairy Protein Research and Technology (WCDPRT) was very active during its 1992 - 1993 operating year. These activities included:

- continuation of important dairy products/processing research projects;
- holding the WCDPRT Annual Meeting on June 30, 1993 at Utah State University;
- communicating to the dairy industry the emerging technologies and research results;
- reporting many project results of WDFRC projects at scientific meetings;
- Technical Advisory Committee (TAC) review of newly proposed research projects.

Twelve (12) research projects were active during this past year. Some research projects came to completion during this time. The three (3) research areas of the WDFRC had active projects. Many projects were actively continuing into the next operating year (1993-1994).

The WDFRC Annual Meeting was held on June 30,, 1991 on the Utah State University campus. The Operational Advisory Committee (OAC) of the WCDPRT provided very significant input into the future direction and focus of the WDFRC. At this OAC meeting, update report of all WDFRC-sponsored projects were presented.

The OAC accepted the resignation of Dr. Paul Savello as director of the WCDPRT and expressed their appreciation for the excellent leadership Dr. Savello provided to the Center. Dr. Donald J. McMahon was installed the new director of the Center.

The TAC reviewed twenty-one (21) new research proposals during March, 1992. Nine (9) proposals were eventually accepted by the Director and the OAC. This year the TAC was made up of three (3) dairy industry representatives, one (1 ) dairy researcher on the faculty of Oregon State University, one (1 ) dairy researcher on the faculty of the University of Minnesota, and the WCDPRT Director. The Director believes that exposing dairy researchers at other institutions (particularly at other NDPRB Dairy Centers) to the research ideas of WCDPRT researchers can lead to meaningful collaborative efforts between Centers. Such collaboration among scientists can prove most beneficial to produce important and timely research results. It is fully expected that having another dairy researcher from another Dairy Center on the WCDPRT TAC will prove to be an excellent vehicle to have researchers "hitchhike" and "brainstorm" new research ideas as well to pool expertise and equipment.

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/transferred to all supportive and contributing parties.

## WESTERN DAIRY FOODS RESEARCH CENTER

### 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  
Arlington VA 22201  
(703) 528-4800

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**WESTERN DAIRY FOODS RESEARCH CENTER**

**OPERATIONAL ADVISORY COMMITTEE**

(continued)

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# WESTERN DAIRY FOODS RESEARCH CENTER

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# WDFRC BUDGET ACTIVITY

## 1993 -1994 FISCAL YEAR

NATIONAL DAIRY PROMOTION AND RESEARCH BOARD		500,000
REGIONAL/INDUSTRY SUPPORT:		
Utah Dairy Commission	50,000	
United Dairymen of Idaho	50,000	
Western Dairy Farmers' Promotion Association	10,000	
Oregon Dairy Products Commission	40,000	
Kraft General Foods, Inc.	5,000	
Schreiber Foods, Inc.	5,000	
Marschall-Rhone Poulenc, Inc.	5,000	
State of Utah Center of Excellence "Center for Dairy Foods Technology"	150,000	
TOTAL REGIONAL/INDUSTRY SUPPORT	315,000	
FY94 TOTAL DAIRY RESEARCH CONTRIBUTIONS		\$815,000
FY93 BALANCE FORWARD		87,787
FY94 COMMITTED RESEARCH FUNDS		
FY 94 RESEARCH	(454,064)	
ADMINISTRATIVE	(50,000)	
CENTER OF EXCELLENCE	(150,000)	
TOTAL COMMITTED RESEARCH FUNDS		(654,064)
FY94 BALANCE FORWARD		\$248,723

## 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:** Identifying Batch of Origin of Finished Cheese Made in Continuous Processes

**Personnel:** Lynn V. Ogden, Dept. of Food Science and Nutrition.  
Brigham Young University

Salvador U. Parco, Dept FSN, BYU

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

**Objectives:**

Use of cheddaring and blockforming towers in cheese manufacturing has considerably increased the efficiency of cheese plants. A serious quality control problem created by this semi-continuous process is difficulty in tracking batches. Current equipment and procedures for tracking batch of origin require that all towers be full at the end of the transfer of each batch to the towers and that all towers run at the same rate. Many plants can not use the system because of the impracticallity of those conditions. When one batch is substandard and batch tracking is poor, the equivalent of three batches are downgraded to assure that all of the substandard cheese is included when the Commodity Credit Corporation buys cheese. When selling to the private sector the problem is similar. Either excessive numbers of blocks have to be recalled and checked to identify the substandard cheese or excessive numbers of blocks are sold as distressed cheese to avoid expensive checking. The potential is high that some low quality cheese will make it on the market and damage the products reputation.

The objective of this project is to develop a system to track batches through pressing towers. We were to evaluate the suitability of the Hunter Qual Probe as a sensor and turmeric or additional annatto as a marker in alternate batches of cheddar cheese. If feasible, it is a further objective to develop a system applying the concept. If not feasible, it is a further objective to search for and assess other sensor technologies for this application.

**Results:**

Less than acceptable reliability using the colorimetric approach has reported in 1992 and attention was turned to assessment of other sensor approaches. The reliability of two commercially available Ultrasonic instruments in partially evacuated environment were evaluated. One of the instruments read distances reliably to 5.8 meters at 381 mm Hg vacuum. The Ultrasonic instruments did not perform as well in the actual tower. Although the vacuum was less than 254 mm Hg, distances readings became unreliable at greater distances than 10 feet. We concluded that ultrasonic sensors would not be reliable enough for this application. Concern about the lack of compatibility with the harsh conditions inside the tower also raised doubts as to the durability of such a system.

A thermal dissipation approach was conceived and explored in which all sensors would be outside the tower. Mounted outside the inner skin of the tower, resistance heaters and thermistors sense how quickly applied heat is dissipated from the stainless steel skin. More rapid dissipation

indicates that cheese is on the inside and is conducting heat away. A buildup of heat indicates that no cheese (heat dissipating mass) is on the other side of the skin. A string of prototype sensors were fabricated and mounted on the outside of a stainless steel milk can. They were able to differentiate the presence of absence of water in the can and a light display indicated the level of water in the can. It is expected that a computerized system that scans a string of sensors and measures the rate of temperature rise using very short time increments and very small currents, could be a very reliable sensor and could be linked to a computerized control system that could not only track batches but also control the depth of cheese in the tower.

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

Inability to accurately identify the batch of origin of specific units of cheese for recall or downgrading is a problem in plants using cheddaring and/or block forming towers in cheese manufacturing. One plant manager estimated the unnecessary value reduction on his cheese to be \$250,000 per year assuming that 6.5 million pounds of cheese was unnecessarily downgraded and an average reduction in value of \$.04 per pound. Ability to identify batch of origin would prevent that loss.

#### **Publications:**

Parco, S.U. and L.V. Ogden. 1992. Batch determination in semi-continuous cheesemaking operations using on-line colorimetry. *J. Dairy Sci.* 75(supp 1): 127.

**Project Title:** Comparative Effects of Whey Protein Concentrate and Phosphate on Bind and Acceptability of Turkey Rolls

**Personnel:** Daren Cornforth, Department of Nutrition & Food Sciences, Utah State University

Brent Dobson, Department of Nutrition & Food Sciences, Utah State University

Igor Moiseev, Department of Nutrition & Food Sciences, Utah State University

**Funding:** Western Center For Dairy Protein Research And Technology

**Objectives:**

Phosphates (primarily sodium tripolyphosphate) are widely used to increase the bind strength and cooked yield of processed meat products. However, phosphates are relatively expensive, and are slow to dissolve in brines. There is also evidence that phosphates in foods reduce the absorption of zinc, calcium, and iron (Mahoney and Hendricks, 1978; Zemel and Bidari, 1983). Phosphates are not permitted as ingredients in cooked poultry products imported into Japan. Thus, there is some interest in the development of alternative binding agents for cooked meat products. Milk proteins have potential as alternative binding agents in cooked meats. We have previously shown that both nonfat dry milk and whey protein concentrate will increase the bind strength of turkey rolls, compared to controls made without milk proteins (Dobson et al., 1993). Thus, the objective of this study was to compare bind strength of turkey rolls made with phosphate or with various commercially available whey protein concentrates.

**Results:**

Turkey rolls (90% breast meat, 10% thigh meat, 10% added water, 1% salt ) were made with no WPC or phosphate (control), sodium tripolyphosphate (0.5%), or WPC (1 or 3%). All percentages were based on meat weight. Three types of WPC were compared;

- WPC-50 (50 % protein)
- WPC-60 (60% protein, acidified to pH 4.5)
- WPC-75 (75% protein, pH 7.0, "high-gel")

Rolls made with phosphate had significantly higher bind strength and firmer texture than controls or rolls made with WPC. Rolls made with 1%WPC-75 also had higher bind strength than controls. Rolls made with 1% WPC-60 had the lowest bind strength and cohesiveness. Rolls with 1% WPC-50 or 1% WPC-75 had moderate turkey flavor. In general, rolls made with 3% WPC had lower scores for intensity of turkey flavor. We have previously shown that nonfat dry milk inhibits the pink discoloration that sometimes develops during refrigerated storage of turkey rolls

(Dobson and Cornforth, 1992). In this study however, no color differences were observed among treatments and controls.

### **Conclusions:**

Use of high-gel WPC produced rolls with higher bind and acceptability than controls, but not as high as rolls made with phosphate. WPC-50 and acidified WPC (WPC-60) were unacceptable as binding agents in turkey rolls.

### **Publications:**

Dobson, B. N. and Cornforth, D. P. 1992. Nonfat dry milk inhibits pink discoloration in turkey rolls. *Poultry Sci.* 71:1943.

Dobson, B. N., Moiseev, I. V., Cornforth, D. P., Savello, P., Wood, R. J. and Andersen, R. 1993. An instrument for measuring bind strength of restructured and emulsion-type meat products. *J. Texture Studies.* In press.

Mahoney, A. W. and Hendricks, D. G. 1978. Some effects of different phosphate compounds on iron and calcium absorption. *J. Food Sci.* 43:1473.

Zemel, M. B. and Bidari, M. T. 1983. Zinc, iron, and copper availability as affected by ortho phosphates, polyphosphates and calcium. *J. Food Sci.* 48:567.

## FINAL REPORT

**Project Title:** Evaluation of Iron-Protein Complexes in Iron-Fortified Dairy Products

**Personnel:** Dr. Arthur W. Mahoney (deceased)

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  
Utah Agriculture Experiment Station

**Project Period:** July 1, 1989 to December 31, 1992

### Objectives:

The primary objective of this study is to determine the chemistry of iron-protein complexes in milk as related to cheese making. The mechanism and thermodynamics of iron-protein complex formation is necessary to understand the functional and stability characteristics of iron-milk protein complexes. Elucidation of the mechanism would provide the information to enable the dairy processor to use conditions and/or competitive reagents to maximize complex formation. The specific objectives of the project are to:

1. To determine the nature of interaction of individual milk proteins, viz.,  $\alpha_{S1}$ -casein,  $\beta$ -casein,  $\kappa$ -casein,  $\beta$ -lactoglobulin,  $\alpha$ -lactalbumin, and bovine serum albumin with ferric iron at pH 6.60 and possible side chain residues involved in the complex formation.
2. To study the effect of pH, NaCl concentration, Ca(II), and dephosphorylation of protein on the binding of iron(III) to  $\alpha_{S1}$ -casein (major casein). Effect of pH on the possible side chain residues involved in the complex formation.
3. To determine the effect of iron binding on the conformation and self-aggregation of different milk proteins at pH 6.60 and at different pHs for  $\alpha_{S1}$ -casein.
4. To study the effect of ferric chloride on the chymosin hydrolysis and rennet clotting time of milk.
5. To study the effect of ferric chloride addition to milk on the physico-chemical properties of casein micelles, such as, iron(III), calcium and phosphorous content, voluminosity, and surface hydrophobicity.
6. To determine the catalytic potency of iron-milk protein complexes on oxidative damage to model lipids.



## Methodology:

1. Binding of iron(III) to milk proteins was measured by diafiltration method using Fe(III)-Nitrilotriacetic Acid(NTA).
2. Possible iron(III)-binding groups in proteins were determined by monitoring visible-difference spectra of iron(III)-protein complexes in the visible region (330 to 700 nm).
3. Iron(III)-induced conformational changes in the proteins were monitored by ultraviolet-difference spectroscopy and fluorescence spectroscopy, and iron(III)-induced aggregation of proteins was monitored by FPLC on Superose-6 gel filtration column (30 x 1.0 cm).
4. Chymosin hydrolysis in milk was followed by monitoring the release of macropptide and rennet clotting time by Formagraph.
5. Calcium and iron were determined by Atomic Absorption Spectroscopy.

## Results:

**Objective 1: Binding of Iron(III) to  $\alpha_{S1}$ -Casein,  $\beta$ -Casein,  $\kappa$ -Casein,  $\beta$ -Lactoglobulin,  $\alpha$ -Lactalbumin, and Bovine Serum Albumin at pH 6.60.** Binding of iron(III) to individual protein fractions was studied by diafiltration method and binding isotherms are shown in Figure 1. Binding data analyzed by Scatchard equation are given in Table I. Binding of iron(III) to all the proteins studied increased as the free iron(III) concentration increased. Scatchard analysis indicated that both casein and whey protein fractions (except  $\beta$ -LG and  $\alpha$ -LA) have two groups of non-identical binding sites with differing affinities for binding iron(III). It appears that first set of binding sites ( $n_1$ ) are preferentially filled, compared to the second set of binding sites ( $n_2$ ). The values of free energy change ( $\Delta G$ ) calculated using the relationship,  $\Delta G = -RT \ln K$ , for different iron(III) binding sites in casein and whey protein fractions were negative and low in magnitude (Table I), indicating that the complex formation between iron(III) and protein fractions is spontaneous and thermodynamically favorable. Relative binding of iron(III) to different casein and whey protein fractions follows the order:  $\alpha_{S1}$ -CN >  $\beta$ -CN > BSA >  $\kappa$ -CN >  $\beta$ -LG >  $\alpha$ -LA.

**Visible-Difference Spectra of Iron(III) - Protein Complexes at pH 6.60.** Difference absorption spectra of iron(III)-protein complexes in PIPES (10mM)-NaCl (100mM) buffer, pH 6.60 were carried out in order to determine the possible amino acid side chain groups involved in the binding of iron(III) to different proteins. Difference absorption spectra of iron(III)- $\alpha_{S1}$ -CN and iron(III)- $\beta$ -CN complexes have negative absorption bands in 420-421nm region and positive absorption bands in 470-471nm region, 491-492 nm region, and in 560-562 nm region ; iron(III)- $\kappa$ -CN complexes have positive absorption bands in 423-425 region, 470-471nm region, 491-492 nm region and 560-562 nm region; and iron(III)-BSA, iron(III)- $\beta$ -LG and iron(III)- $\alpha$ -LA complexes have positive absorption bands in 421-422 nm region, 470-471 nm region, 491-492 nm region, and 568-570 nm region. The model spectra of the complexes of phosphorylserine, Asp, Glu and other amino acids with iron(III)

revealed that the negative absorption band in 420 nm region was due to phosphorylserine-iron(III) complexes and the positive band in 565 nm was due to and carboxyl-iron(III) complexes, where as the positive bands in 420, 470 and 490 nm region were possibly due to a chelate site involving carboxyl, nitrogen and oxygen groups. This may indicate that phosphoserine groups are the major iron(III) binding sites in  $\alpha_1$ -CN and  $\beta$ -CN, where as carboxyl groups are the major iron(III) binding sites in  $\kappa$ -CN, BSA,  $\beta$ -LG, and  $\alpha$ -LA.

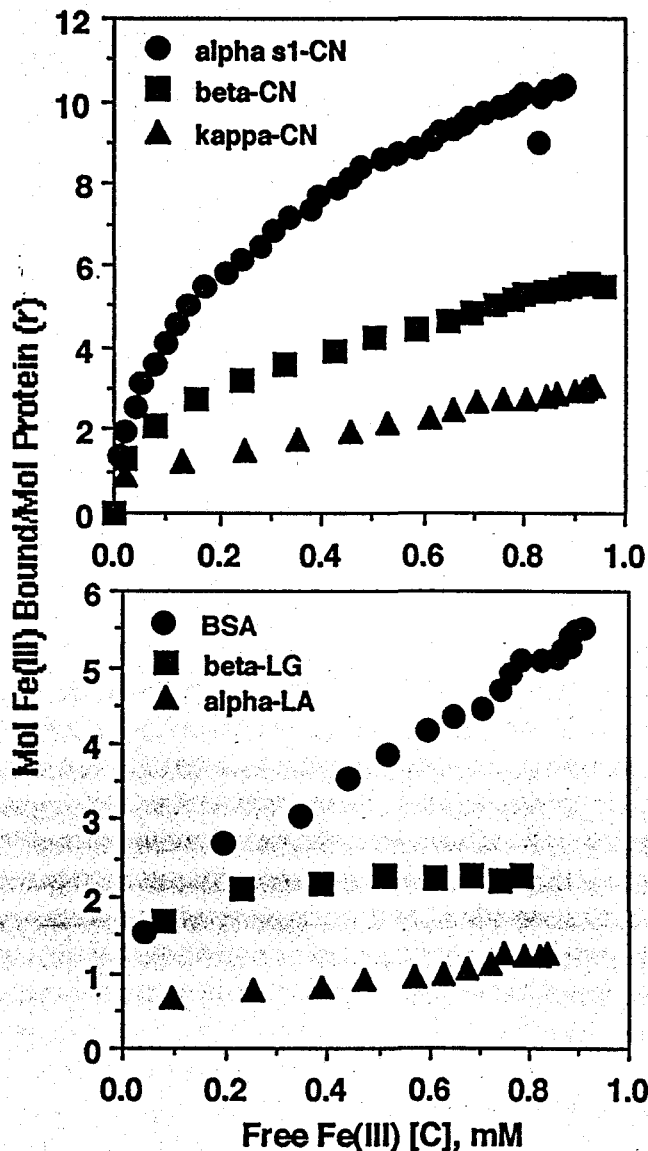


Fig. 1A. Binding Curves of Fe(III) to Caseins, pH 6.60,  $\mu=0.1$

Fig. 1B. Binding Curves of Fe(III) to Whey Proteins

**Objective 2: Effect of pH, NaCl, Ca(II) and Dephosphorylation of Protein on the Binding of Fe(III) to  $\alpha_1$ -Casein.** Binding of iron(III) to  $\alpha_1$ -casein was studied as a function of pH (5.6, 6.1, 6.6, 7.2, and 7.8), NaCl concentration (0.1 and 0.5 M), and dephosphorylation of protein using diafiltration method and the binding data analyzed by Scatchard equation. pH and NaCl had no influence on the number of iron binding sites on the protein, which remained constant at  $n=20$  (Table II). However, binding affinity of iron(III) to protein decreased with an increase in pH from 5.6 to 7.8 and NaCl from 0.1 to 0.5 M (Table 2). Thus, from the practical point of view, the binding affinity of iron(III) increases as

the pH of milk is lowered by microbial action during cheese making. Dephosphorylation of  $\alpha_{S1}$ -casein decreased the number of iron binding sites on the protein (Table 2) indicating that phosphoserine groups play a major role in the binding. In another experiment, partial displacement of protein-bound Ca(II) by iron(III) was observed for  $\alpha_{S1}$ -casein. This indicates that iron(III) successfully competes for some of the Ca(II) binding sites on the protein. Free energy change ( $\Delta G = -RT \ln K$ ) calculated for the binding of iron(III) to  $\alpha_{S1}$ -casein under different pHs and NaCl concentrations was negative and low in magnitude (-3.79 and -7.28 k cal M<sup>-1</sup>) indicating that the binding of iron(III) to protein is instantaneous and thermodynamically favorable.

Table I. Thermodynamic Parameters for the Binding of Iron(III) to Milk Proteins (pH 6.6,  $\mu=0.1$ , 24°C)

Protein	Fe to protein molar ratio	Number of binding sites (n)	Binding constants (K) (M <sup>-1</sup> x 10 <sup>3</sup> )	Free energy change ( $\Delta G$ ) (k. cal. M <sup>-1</sup> )
$\alpha_{S1}$ -CN	4.7	14.0 (2.7, 4.5, 6.8)	128 to 2.9	-6.9 to -4.7
$\beta$ -CN	4.8	9.0 (2.7, 2.0, 4.3)	44 to 1.3	-6.3 to -4.2
$\kappa$ -CN	3.8	6.0 (1.5, 4.5)	88 to 1.0	-6.7 to -4.1
BSA	13.3	8.5 (3.5, 5.0)	20 to 1.8	-5.8 to -4.4
$\beta$ -LG	3.7	2.4	31	-6.1
$\alpha$ -LA	2.8	0.9	32	-6.1

**Effect of pH and Dephosphorylation on Visible-Difference Spectra of Iron(III) -  $\alpha_{S1}$ -Casein Complexes.** Difference absorption spectra of iron(III)-protein complexes in the visible region (370 to 750 nm) were carried out to determine the possible amino acid side chain groups involved in the binding of iron(III) to  $\alpha_{S1}$ -casein at different pHs (5.6 to 7.8). Negative absorption band in 420 nm region, and positive absorption bands in 470, 490 and 560 nm region were observed for the protein at all pHs. The model spectra of the complexes of iron(III) with phosphorylserine, Asp, Glu and other amino acids revealed that the negative absorption band in 420 nm region was due to iron(III)-phosphorylserine complexes and the positive band in 565 nm region was due to iron(III)-carboxyl complexes, where as the positive bands in 420, 470 and 490 nm region were possibly due to a chelate site involving carboxyl, nitrogen and oxygen groups. When  $\alpha_{S1}$ -casein was dephosphorylated the negative absorption band in 420 nm region disappeared and a positive absorption band appeared in its place, further confirming that the negative absorption band in 420 nm region is contributed by iron(III)-phosphorylserine complexes. The magnitude of the negative absorption band in 420 nm region decreased, where as the positive absorption band in 470, 490, and 565 nm region increased with an increase in pH. This may indicate that the involvement of carboxyl groups in the complex formation increased as the pH is moved away from their isoelectric pH. Thus, phosphorylserines and carboxyl groups of Asp and Glu seem to play a major role in the binding of iron(III) by  $\alpha_{S1}$ -casein.

Table II. Thermodynamic Parameters for the Binding of Iron(III) to  $\alpha_{S1}$ -Casein at 24°C

Fe to Protein Molar Ratio	pH	NaCl (mM)	Number of Binding Sites ( <i>n</i> )	<i>K</i> (M <sup>-1</sup> ) x 10 <sup>3</sup>	$\Delta G$ (k. cal. M <sup>-1</sup> )
9.4	5.6	100	20 (8.0, 7.0, 5.0)	22 to 2.5	-5.9 to -4.6
9.4	6.1	100	20 (7.7, 12.3)	8 to 1.7	-5.3 to -4.4
9.4	6.6	100	20 (5.2, 4.8, 10)	46 to 1.0	-6.3 to -4.1
9.4	7.2	100	20 (4.8, 15.2)	24 to 1.1	-5.9 to -4.1
9.4	7.8	100	20 (4.6, 15.4)	21 to 0.6	-5.9 to -3.8
4.7	6.6	100	14 (2.7, 4.5, 6.8)	128 to 2.9	-6.9 to -4.7
4.7	6.6	500	14 (2.2, 4.5, 7.3)	227 to 2.0	-7.3 to -4.5
9.4	7.8*	100	9 (4.0, 5.0)	35 to 1.2	-6.2 to -4.2

\*Dephosphorylated  $\alpha_{S1}$ -casein

**Objective 3: Iron(III)-Induced Conformational Changes in  $\alpha_{S1}$ -Casein,  $\beta$ -Casein,  $\kappa$ -Casein,  $\beta$ -Lactoglobulin,  $\alpha$ -Lactalbumin, and Bovine Serum Albumin at pH 6.60.** Conformational changes in proteins, especially, changes in the environment of aromatic side chains in the proteins as a result of binding of iron(III) were monitored by following fluorescence emission after excitation at 280 nm. Addition of iron(III) caused a decrease in fluorescence emission intensity together with a red shift of the emission maximum for the proteins, except  $\beta$ -lactoglobulin. Iron(III) addition to  $\beta$ -lactoglobulin caused a decrease in emission intensity without affecting the emission maximum. The results indicate that binding of iron(III) to proteins perturbed the environment of aromatic amino acid (Trp and Tyr) residues possibly by exposing them to a more polar environment.

Since, binding of metal ions (Ca, Mg, Ba, Zn) to caseins has been reported to induce aggregation of proteins, it was of interest to see if iron(III) induces similar changes in milk proteins. Therefore, Iron(III)-induced aggregation of  $\alpha_{S1}$ -casein,  $\beta$ -casein,  $\kappa$ -casein,  $\beta$ -lactoglobulin,  $\alpha$ -lactalbumin, and bovine serum albumin at pH 6.60 was followed by FPLC on Superose-6 gel filtration column (30 x 1.0 cm). The results indicated that the binding of iron(III) to milk proteins did not induce aggregation. This may be due to the differences in the nature of binding of iron(III) (which forms co-ordinate complexes) to proteins compared to other bivalent metal ions (which form ionic complexes).

**Effect of pH on Iron(III)-Induced Conformational Changes in  $\alpha_{S1}$ -Casein.** Intrinsic fluorescence studies at pH 6.60 indicated that iron(III) binding to  $\alpha_{S1}$ -casein perturbed the environment of aromatic amino acid residues in the protein. Therefore, the iron(III)-induced conformational changes in the protein were further probed at different pHs by following UV-difference spectra and fluorescence emission spectra. The UV-difference spectra of  $\alpha_{S1}$ -casein induced by iron(III) at pH 5.6, 6.1, 6.6, 7.2, and 7.8 had absorption bands at 310 nm indicating the possible involvement of tryptophan residues in charge-transfer type

complex formation with iron(III). Since it is characteristic of charge-transfer type complexes to quench tryptophan fluorescence in proteins, the accessibility of tryptophanyl residues in  $\alpha_{S1}$ -casein in the absence and presence of iron(III) was followed by iodide quenching at pH 6.6. Intrinsic tryptophan fluorescence of  $\alpha_{S1}$ -casein excited at 295 nm was quenched by iodide indicating the partial exposure of tryptophan residues in protein. Addition of iron(III) to protein caused further quenching of tryptophanyl fluorescence by iodide. This further supports the UV spectral data that binding of iron(III) to protein brought about changes in the immediate vicinity of tryptophan environment. This could be due to the involvement of tryptophan residues in charge-transfer type complex formation with iron(III), thus exposing them to a more polar environment. Addition of Ca(II) to  $\alpha_{S1}$ -casein induces progressive aggregation as shown by a net increase in fluorescence emission intensity and a blue shift in fluorescence maximum. However, iron(III) does not seem to induce any aggregation, instead seems to form soluble iron(III)-protein complexes.

Fluorescence emission of  $\alpha_{S1}$ -casein (excited at 280 nm) at different pHs (5.6 to 7.8) was followed as a function of iron(III) concentration (0 to 0.2 mM). Addition of increasing concentrations of iron(III) brings about a red shift of the emission maximum, together with a decrease in fluorescence intensity at all pHs. Fluorescence intensity of  $\alpha_{S1}$ -casein as a function of iron(III) concentration was fitted to a modified Stern-Volmer plot and quenching constant ( $K_Q$ ) was calculated from the slope. Quenching constant was high at pH 5.6 ( $1.1 \times 10^4 \text{ M}^{-1}$ ) and decreased with an increase in pH ( $0.65 \times 10^4 \text{ M}^{-1}$  at pH 7.8). This indicates that iron(III) binding had maximum effect on the conformation of protein at pH 5.6 than at pH 7.8. Since conformational changes in general affect functional properties of proteins, it was of interest to see if binding of iron(III) affects Ca(II) sensitivity of  $\alpha_{S1}$ -casein; addition of iron(III) to protein increased its Ca(II) sensitivity.

**Objective 4: Effect of Ferric Chloride on Chymosin Hydrolysis and Rennet Clotting Time of Milk.** Since binding of iron(III) to caseins induced conformational changes, it was of practical interest to see if addition of ferric chloride to milk during cheese making would affect renneting properties. Therefore, ferric chloride (0 to 0.86 mM, about 0.2 mM concentration is normally employed making iron-fortified Cheddar cheese) was added to cold milk before pasteurization and to pasteurized at 30°C (as in the case of regular cheese making process) and chymosin hydrolysis and rennet clotting time (RCT) were evaluated. Iron(III) at all the concentrations employed had no effect on the chymosin hydrolysis of either milks. However, iron(III) decreased the RCT of both whole and skim milk when added to cold milk before pasteurization and increased when it was added to pasteurized skim milk, but it barely affected the RCT of pasteurized whole milk.

**Objective 5: Effect of Ferric Chloride Addition to Milk on the Iron, Calcium and Inorganic Phosphorous Contents, Voluminosity, and Surface Hydrophobicity of Casein Micelles.** Ferric chloride at 0, 0.43 and 0.86 mM concentration was added to cold milk before pasteurization and to pasteurized milk under constant stirring and allowed to equilibrate for 30 min. The milk was then centrifuged at 100,000 x g to partition the casein micelles from whey and the iron, calcium and phosphorous contents and voluminosity of micelles was determined. Surface hydrophobicity of casein micelles was determined by adding 0.2 mM ANS to milk before centrifugation and by estimating the distribution of ANS

in micellar and whey phases by measuring ANS fluorescence. Addition of ferric chloride to either milks resulted in a decrease in voluminosity and calcium content, and an increase in iron and inorganic phosphorous content of micelles with an increase in ferric chloride concentration in milk. However, addition of ferric chloride to milk before pasteurization increased the surface hydrophobicity of micelles and addition of ferric chloride to milk after pasteurization decreased the surface hydrophobicity of micelles. This may explain why RCT of milk decreased when ferric chloride was added to milk before pasteurization, and it increased when ferric chloride was added to milk after pasteurization.

**Objective 6: Catalytic Potency of Iron-Milk Protein Complexes on Oxidative Damage to Model Lipids.** Off-flavor production due to iron-induced lipid peroxidation is a major concern in fortifying dairy foods with iron. Hence, it is of practical interest to know the catalytic potency of iron(III)-casein and iron(III)-whey complexes on iron-catalyzed lipid peroxidation. Therefore the inhibitory effect of casein and whey protein fractions on iron-catalyzed lipid peroxidation was studied in soy lecithin-iron(III)-ascorbate model system by estimating TBARS (nmol MDA per mg lipid). The results indicated that both casein and whey protein fractions inhibited iron-induced lipid peroxidation in proportion to their iron(III)-binding capacity.

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

Iron fortification would increase the iron intakes of people who consume large amounts of dairy products, and it would allow people concerned with their nutriture to consume larger amounts of dairy products to achieve greater calcium intakes. Thus, dairy products would be even more healthful in the diet if iron-fortified. This research will provide basic information on the mechanisms of iron binding to proteins in dairy products, information essential to industrializing the technology of fortifying dairy products with iron.

#### **Publications:**

Reddy, I. M. and Mahoney, A. W. Effect of  $\text{FeCl}_3$  on chymosin hydrolysis and rennet clotting time of milk. *J. Dairy Sci.* 75, 2648, 1992.

Reddy, I. M. and Mahoney, A. W. The effect of iron on the renneting of milk. *J. Dairy Sci.* 74 (Suppl. 1), 100, D-57, 1991.

Reddy, I. M. and Mahoney, A. W. Binding of  $\text{Fe(III)}$  to bovine  $\alpha_{S1}$ -casein. *J. Dairy Sci.* 74 (Suppl. 1), 100, D-58, 1991.

Reddy, I. M. and Mahoney, A. W. A study of the interaction of  $\text{Fe(III)}$  to bovine  $\alpha_{S1}$ -casein using ultraviolet and fluorescence spectroscopy. *J. Dairy Sci.* 74 (Suppl. 1), 100, D-59, 1991.

Reddy, I. M. and Mahoney, A. W. Characterization of  $\text{Fe(III)}$  - milk protein complexes. *In* Brief Communications and Abstracts of Posters of the XXIII International Dairy Congress, Vol. I, 253, P-471, 1990.

## **Publications (Contd.)**

Reddy, I. M. and Mahoney, A. W. (1993). Diafiltration and visible spectroscopic study of the binding of iron(III) to bovine  $\alpha_{S1}$ -,  $\beta$ -, and  $\kappa$ -caseins, bovine serum albumin,  $\beta$ -lactoglobulin, and  $\alpha$ -lactalbumin at pH 6.60. (In Review)

Reddy, I. M. and Mahoney, A. W. (1993). A visible spectroscopic study of iron(III)-amino acid complexes at pH 6.60. (In Review)

Reddy, I. M. and Mahoney, A. W. (1993). Iron(III)-induced conformational changes in bovine  $\alpha_{S1}$ -,  $\beta$ -, and  $\kappa$ -caseins, bovine serum albumin,  $\beta$ -lactoglobulin, and  $\alpha$ -lactalbumin at pH 6.6 studied by ultraviolet and fluorescence spectroscopy. (In Preparation)

Reddy, I. M. and Mahoney, A. W. (1993). Effect of pH, NaCl, CaCl<sub>2</sub>, and dephosphorylation of protein on the binding of iron(III) to  $\alpha_{S1}$ -casein. (In Preparation)

Reddy, I. M. and Mahoney, A. W. (1993). A study of the interaction of iron(III) to bovine  $\alpha_{S1}$ -casein using ultraviolet and fluorescence spectroscopy. (In Preparation)

Reddy, I. M. and Mahoney, A. W. (1993). Inhibition of iron-catalyzed lipid peroxidation by casein and whey protein fractions. (In Preparation)

## **Papers Presented at Conferences:**

Reddy, I. M. and Mahoney, A. W. Effect of Iron on the renneting of milk. Presented at 86th ADSA Annual Meeting, Utah State University, Logan, August 12-15, 1991.

Reddy, I. M. and Mahoney, A. W. Binding of Fe(III) to bovine  $\alpha_{S1}$ -casein. Presented at 86th ADSA Annual Meeting, Utah State University, Logan, August 12-15, 1991.

Reddy, I. M. and Mahoney, A. W. A study of the interaction of Fe(III) with bovine  $\alpha_{S1}$ -casein using ultraviolet and fluorescence spectroscopy. Presented at 86th ADSA Annual Meeting, Utah State University, Logan, August 12-15, 1991.

Reddy, I. M. and Mahoney, A. W. Characterization of Fe(III) - milk protein complexes. Presented at 'The 23rd International Dairy Congress', Montreal, Canada, October 7-12, 1990.

## **Short Courses:**

Dr. Mohan Reddy attended a ACS lecture-laboratory short course on 'Microemulsions, Vesicles, and Liquid Crystals' at Clarkson University, Potsdam, NY during July 19-24, 1992 to develop methodology at Utah State University.

**Theses/Dissertations:** Nil.

**Patents:** Nil.

**Project Title:** Estimation of Individual Milk Proteins and Genetic Variants by Multicomponent Analysis of Amino Acid Profiles

**Personnel:** Rodney J. Brown, Dept. of Nutrition and Food Sciences, Utah State University.

Carol M. Hollar, Dept. of Nutrition and Food Sciences, Utah State University.

**Funding:** Western Dairy Foods Research Center

**Objectives:**

The overall objective is to determine proportions of specific milk proteins, groups of proteins and selected genetic variants of milk and other dairy products using information obtained from amino acid analysis, fast protein liquid chromatography and isoelectric focusing of samples. Completing the following specific objectives will enable the overall objective to be met.

1. Determine concentrations of groups of proteins in milk such as caseins or whey protein and the concentrations of specific milk proteins:  $\alpha_{s1}$ -,  $\alpha_{s2}$ -,  $\beta$ -, and  $\kappa$ -caseins,  $\alpha$ -lactalbumin,  $\beta$ -lactoglobulin, bovine serum albumin using amino acid analysis.
2. Separate genetic variants of specific milk proteins, and use amino acid analysis to quantify individual variants in a protein mixture.
3. Use techniques developed in 1-2 to analyze milk and other dairy products.
4. Determine mathematical procedures to obtain the most accurate and reproducible methods for estimating milk protein concentrations.

**Results:**

Percentages of  $\kappa$ -casein,  $\beta$ -casein,  $\alpha_{s1}$ -casein, and  $\alpha_{s2}$ -casein obtained with isoelectric focusing, cation-exchange fast protein liquid chromatography, and anion-exchange fast protein liquid chromatography compare well with published results. Isoelectric focusing and anion-exchange fast protein liquid chromatography methods separated  $\kappa$ -casein into its A and B variants. Isoelectric focusing and cation-exchange fast protein liquid chromatography separated  $\beta$ -casein into its A<sup>1</sup>, A<sup>2</sup>, and B variants.



Although amino acid analysis can identify whether  $\beta$ -casein A<sup>1</sup>, A<sup>2</sup>, or B variants are present, it cannot identify whether  $\kappa$ -casein A and B variants are present. Stepwise regression equations derived to predict samples as homozygous or heterozygous for  $\kappa$ -casein A and B and  $\beta$ -casein A<sup>1</sup>, A<sup>2</sup>, and B had coefficient of determination values of .18, .58, .82, and .72 for  $\kappa$ -casein A and B,  $\beta$ -casein A<sup>1</sup>,  $\beta$ -casein A<sup>2</sup>, and  $\beta$ -casein B.

### **Impact of Research:**

The goal of this project is to determine proportions of specific milk proteins (down to the level of specific genetic variants) or groups of proteins in milk and other dairy products from the information contained in a single amino acid analysis of a sample. As profit margins get tighter, the more closely milk supplied by farmers meets the requirements of dairy manufacturers becomes more important. Manufacturers prefer receiving milk that results in improved cheese yield and processing characteristics. Developing techniques to separate and quantify caseins and their genetic variants will help provide a more desirable milk supply to manufacturers.

### **Publications:**

Hollar, C. H., R.J. Brown and J. F. Medrano. 1990. Quantification of casein genetic variants by RP-HPLC, PAGE, and amino acid analysis. 85<sup>th</sup> American Dairy Science Association Meeting *J. Dairy Sci.* 73:Supp. 1, 80.

Hollar, C. M., A. J. R. Law, D. G. Dalgleish, and R. J. Brown, 1991, Separation of major casein fractions using cation-exchange fast protein liquid chromatography. *J. Dairy Sci.* 74:2403-2409.

Hollar, C. M., A. J. R. Law, D. G. Dalgleish, and R. J. Brown, 1991, Separation of major casein fractions using cation-exchange fast protein liquid chromatography. 86<sup>th</sup> American Dairy Science Association Meeting *J. Dairy Sci.* 74:Supp. 1, 91.

Hollar, C. M., A. J. R. Law, D. G. Dalgleish, J. F. Medrano, and R. J. Brown, 1991, Separation and quantification of  $\beta$ -casein A<sup>1</sup>, A<sup>2</sup> and B using cation-exchange fast protein liquid chromatography. *J. Dairy Sci.* 74:3308-3313.

Hollar, C. M., A. J. R. Law, D. G. Dalgleish, J. F. Medrano, and R. J. Brown, 1991, Separation of  $\beta$ -casein A<sup>1</sup>, A<sup>2</sup> and B using cation-exchange fast protein liquid chromatography. 86<sup>th</sup> American Dairy Science Association Meeting *J. Dairy Sci.* 74:Supp. 1, 103.

Hollar, C.M. 1992. Estimation of Selected Milk Protein Genetic Variants by Multi-Component Analysis of Amino Acid Profiles. Ph.D. Dissertation, Utah State University, Logan.

Hollar, C. M., and R. J. Brown, 1992, Separation and quantification of  $\alpha_{s1}$ -casein,  $\beta$ -casein A<sup>1</sup>, A<sup>2</sup> and B and  $\kappa$ -casein A and B using isoelectric focusing, and cation-exchange and anion-exchange fast protein liquid chromatography. 87<sup>th</sup> American Dairy Science Association Meeting *J. Dairy Sci.* 75:Supp. 1, 121.

Hollar, C. M., and R. J. Brown, 1993, Quantification of  $\alpha_{s1}$ -casein,  $\alpha_{s2}$ -casein,  $\beta$ -casein A<sup>1</sup>-5P, A<sup>2</sup>-5P, and B-5P, and  $\kappa$ -casein A-1P and B-1P using isoelectric focusing and cation- and anion-exchange fast protein liquid chromatography. Submitted for Publication.

Hollar, C. M., and R. J. Brown, 1993, Neuraminidase treatment of whole casein to improve the separation of  $\kappa$ -casein A-1P and B-1P using anion-exchange fast protein liquid chromatography. Submitted for Publication.

Hollar, C. M., and R. J. Brown, 1992, Estimation of  $\kappa$ -casein A-1P and B-1P and  $\beta$ -casein A<sup>1</sup>-5P, A<sup>2</sup>-5P, and B-5P using multi-component analysis of amino acid profiles. Internal Review Completed.

**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

**Objectives:**

The oxidation of milk causing off-flavors continues to be a problem. 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, but current nutritional management of dairy cows relative to vitamins A and E have a definite impact upon the tocopherol content of milk. The specific objectives of this phase of the study are as follows.

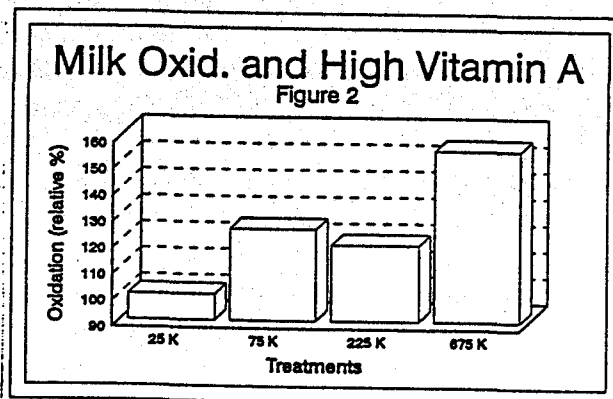
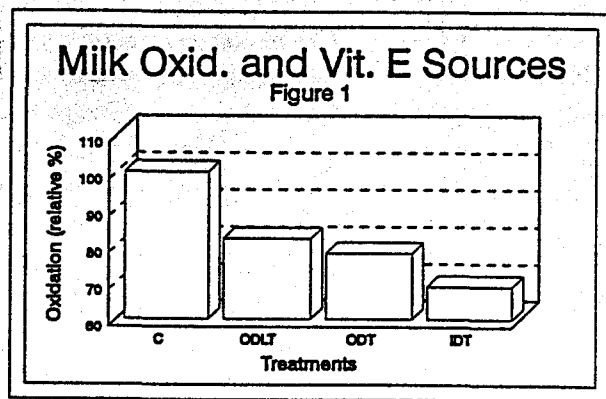
1. To evaluate the effect of various chemical and/or administration forms of vitamin E on milk vitamin E content and milk oxidation.
2. To determine the efficiency of gastrointestinal tract absorption and mammary transfer of vitamin E to milk.
3. To determine the effect of higher dietary levels of vitamin 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 late lactation 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. The milk sampling schedule involved 14 samples collected from day -2 through 22, with the treatments being given on day 0. Both the oral and injectable sources elevated milk alpha-tocopherol by day 1 and peaked on day 2. The oral doses produced elevated levels for 5 day, and the injectable for 9 day. The peak alpha-tocopherol concentrations in the milk at day 2 for treatments C, ODLT, ODT and IDT were .64, .72, .98 and 1.73 mg/l ( $P < .0001$ ). The total alpha-tocopherol secretion in the milk due to the treatments was determined by calculating a baseline for each cow based on average values for day -2, -1, 0, 14 and 22. 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 data indicate a relatively low transfer (3.01 %) of injected d-alpha-tocopherol across the mammary gland into milk. Furthermore, the transfer of vitamin E sources from the digestive tract to milk (.36 and .75 % for dl-alpha-tocopheryl acetate and micellized d-alpha-tocopherol respectively) would indicate a digestive tract absorption of 12.9 and 26.8 % for these two vitamin E sources assuming the same post-absorptive utilization efficiency as for the injectable vitamin E source. The rate of oxidation was measured over an appropriate time period on milk samples with added ferric sulfate by using the thiobarbituric acid (TBA) procedure. Figure 1 shows the decreased oxidation of milk as milk vitamin E is increased via more available vitamin E forms. Relative milk oxidation was significantly ( $P < .01$ ) reduced from 100 % for the control (C) to 82.1, 77.8 and 68.6 % for the oral dl-alpha-tocopheryl acetate (ODLT), oral micellized d-alpha-tocopherol (ODT) and injectable d-alpha-tocopherol (IDT) treatments respectively.



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 124.6, 118.8 and 154.9 % for the medium, high and very high vitamin A treatments respectively.

### **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.* (Abstract).

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*. Adelaide, Australia.

**Project Title:** Use of Cheese Whey for Soil Improvement

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

Darwin Sorensen, Professor and Mark Roginske, Graduate Student, Civil and Environmental Engineering Dept., Utah State University

**Funding:** Western Dairy Foods Research Center

**Objectives:**

Main

This project is a continuation of a project started in 1991. In this aspect of the project we will determine if the optimal application rates for reclamation of sodic and saline-sodic soils is harmful to the environment.

Specific

- Determine the extent crops and soil remove nutrients and COD load from land applied whey.
- Determine the best management practices for utilizing the soluble carbon in the whey as an energy source to reduce nitrate to ammonia and/or N<sub>2</sub> gas as a means of keeping nitrate from entering subsurface waters.
- Determine maximum safe whey land application rate.
- Determine if odor will be a problem in land application of whey and/or how to avoid an odor problem.
- Determine value of whey for land treatment, i.e. from nutrients, pH adjustment, increasing aggregate stability and exposed subsoil reclamation.
- Determine the effects of total salts and salt ratios in whey, on soil physical conditions and on crop growth.

**Results:**

Soil gas samples were collected on 2/16, 2/26, 3/27, 4/17, 6/29, 7/9, and 7/10 for soil oxygen, carbon dioxide, and nitrogen percentage determination by gas chromatography (GC). As of 4/17, CO<sub>2</sub> levels have been between 0.07 and 4.70% with most levels being under 1.00%. Oxygen levels have been between 15.70% and 21.80% and nitrogen levels range from 76.3 to 86.2%. Soil gas samples on 6/29, 7/8, and 7/10 have been run on the GC, but percentages have not yet been computed.

Soil sampling will begin in the fall.

Test Plot Observations Growth on alfalfa plots 1, 2, and 11 receiving the most amount of whey appeared stunted shortly after the 3/29 application. At the time of the first hay cutting (6/15), alfalfa on plots 1, 2, 4, and 12, along with control plot 9, appeared stunted. Remaining plots appeared taller and greener than the rest of the field. At the time of the second hay cutting (7/20), all plots that received whey on 7/8 showed non-uniform growth with some dead alfalfa spots, especially around the instrument areas. Control plots looked similar to the rest of the field.

Whey Application Whey from Cache Valley Cheese was applied to alfalfa test plots as follows:

<u>Date</u>	<u>Plot #</u>	<u>Amount/plot (gal)</u>
1-14-93	1,2,4,6,7,8,10,11,12	196
2-18-93	1,2,4,8,11,12	196
3-29-93	1,2,11	196
7-8-93	1,2,4,6,7,8,10,11,12	196

Alfalfa test plot numbers 3, 5, and 9 are control plots and will not receive whey.

Instrumentation Instruments in the ground at the time of the January, February, and March whey applications were ceramic cups, two at 2 feet and two at 3.5 feet, and soil gas points, one at 2 feet and one at 3.5 feet, for each plot. Four redox probes were installed in each plot at a depth of 30 cm by the time of the July whey application. Oxygen probes will not be installed in any of the plots and the oxygen probe order has been cancelled. Thermistors have been received and will be installed as soon as possible.

Sample Collection and Testing Soil solution samples were collected on 2/13, 2/24, 3/25, 4/16, 6/29, and 7/10 for COD, total Kjeldahl-N,  $\text{NH}_4\text{-N}$ ,  $\text{NO}_3\text{-N}$ , total phosphorus,  $\text{PO}_4\text{-P}$ , iron, manganese,  $\text{SO}_4$ , and  $\text{Cl}^-$  testing. Standard curve generation for  $\text{NO}_3\text{-N}$ ,  $\text{PO}_4\text{-P}$ ,  $\text{SO}_4$ , and  $\text{Cl}^-$  testing by ion chromatograph (IC) has been unsuccessful because of a leaking pump on the IC. A part has been ordered and sample testing will begin as soon as a proper standard curve can be made. Acceptable low level COD standard curves have been generated in replicate and sample testing will begin this week. Generation of an acceptable ferrous iron standard curve by the ferrozine colorimetry method has not been successful. Problems have been narrowed to dirty glassware and are being corrected. Manganese, total iron, total phosphorus, total Kjeldahl-N, and  $\text{NH}_4\text{-N}$  testing will be initiated as time allows.

Impact of Research:

Whey, particularly acid whey, has been considered a by-product of cheese manufacture with value that fluctuates widely with market conditions. Even at the present time, a high percentage of whey is thrown away. The goal of this project is to develop a new market for whey. Use of whey as a soil amendment and fertilizer coincides with the Low Input Sustainable Agriculture (LISA) goals.

Whey can be utilized to reclaim unproductive soils. Technology exists to reclaim sodic and saline sodic soils with expensive chemicals costing up to \$1,000.00/acre. Therefore, land owners can often buy new land for less than it will cost to reclaim these soils. The salt content and pH of acid whey, combined with its fertilizer value makes this an ideal material to reclaim these soils and provides a new market for a dairy product. The value of whey will be enhanced by creating this new demand and at the same time decrease its disposal by undesirable methods.

One of the most attractive aspects of this project is the cooperation between the Idaho dairy industry, Utah State University and the USDA-ARS Soil and Water Management Research Unit, Idaho. This project is receiving high visibility in Idaho and will serve as an example of what the WCDPRT does for the dairy farmer.

The information gleaned from this project will also make it possible to legally apply whey to land. Currently, many states including Idaho and Utah limit to a great extent land application of whey because of lack of application guidelines. Whey must either be dried or treated using some other, often expensive method so it can be sold or considered as wastewater and disposed of at considerable expense because of its high biochemical oxygen demand and solids content.

#### **Publications:**

Jones, S.B., C. W. Robbins, C.L. Hansen. 1993 Sodic Soil Reclamation Using Cottage Cheese (Acid) Whey. *Arid Soil Research and Rehabilitation* 7:51-61.

Jones, S.B., C.L. Hansen, C. W. Robbins. 1993 Fate of Chemical Oxygen Demand from Cottage Cheese (Acid) Whey Applied to a Sodic Soil. *Arid Soil Research and Rehabilitation* 7:71-78.



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

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

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

**Objectives :**

(brief statement on the overall goal of the project)

1. To produce low-fat and non-fat iron-fortified yogurt using FeCl<sub>3</sub>, Fe-casein or Fe-whey protein complex as the iron sources.
2. To determine growth and viability of Lactobacillus delbruekii ssp. bulgaricus and Streptococcus salivarius ssp. thermophilus in iron-fortified yogurt.
3. To determine iron binding sites using Elemental Filtering Electron Microscopy.
4. To determine the best procedure for making a high-quality iron-fortified yogurt.

**Results :**

**Objective 1.** Low-fat (2%) and non-fat iron-fortified yogurt with three sources FeCl<sub>3</sub>, Fe-casein and Fe-whey protein at three levels (10, 20, and 40 mg/Kg) for each sources were successfully made. The two iron sources, Fe-casein and Fe-whey protein were prepared by adding .5 M FeCl<sub>3</sub> into skim milk and cottage cheese whey respectively. The Ferrozine assay was used to determine percent iron recovery of the iron sources. The percent iron recovery of Fe-casein and Fe-whey protein were 46.76 and 88.6 respectively.

**Objective 2.** The yogurt cultures were obtained from Heart to Heart corporation. Enumeration of L. delbruekii ssp. bulgaricus and S. salivarius ssp. thermophilus were done using MRS (pH 5.4) and M17 media respectively. Lactic acid counts for non-fat iron fortified yogurt (FeCl<sub>3</sub>, Fe-casein and Fe-whey protein at 10, 20, 40 mg/Kg) started with 10<sup>8</sup> and in some cases 10<sup>9</sup> CFU/ml for each bacteria. Their number decreased only slightly after one month of storage. There were no significant

differences in bacterial counts between iron-fortified yogurt and the control. The bacterial counts for low fat iron-fortified yogurt is still in progress.

**Objective 3.** Several samples of iron-fortified yogurt were prepared for Scanning Electron Microscopy (SEM). Results indicated that preparation procedure for SEM did not wash away iron and localization of the bound iron was possible. However, Transmission Electron Microscopy (TEM) is necessary for more detail study of bound iron. The initial sample preparation for TEM which involved fixation with glutaraldehyde and osmium tetroxide and embedding with Standard Mollinbauer Araldite mixture (EPON) did not maintain iron in a binding state and localization of iron was not possible. Therefore, further investigations are required for obtaining the best procedure to keep the bound iron in the sample. This may involve utilizing different fixative, dehydrating reagent, transition reagent, embedding media, etc. Our goal is to focus on obtaining an optimum condition which would not cause removal of iron from the sample.

#### **Objective 4.**

**I. Iron Analysis :** The Ferrozine assay was used to quantitate iron in the iron-fortified yogurt after complete wet-ashing with nitric acid and Hydrogen Peroxide. The results from non-fat iron-fortified yogurt indicate uniform distribution of iron throughout the yogurt matrix for all of the sources ( FeCl<sub>3</sub>, Fe-casein and Fe-whey protein). The Analysis of low-fat iron fortified yogurt is still under investigation.

**II. Thiobarbituric Acid Test :** Initially distillation method was used to quantitate lipid oxidation materials in iron fortified yogurt. However, due to reaction of thiobarbituric acid with yogurt components (phospholipids, protein residues, sugar, etc.), an intense yellow color was present in all distillates. According to some investigators, the indicator 'pink' color could be extracted with cyclohexanol, but in case of yogurt, the yellow color was also extracted into cyclohexanol layer. Therefore, a new spectrophotometric method was used to determine lipid oxidation in the yogurt. The results indicates no significant differences between iron-fortified yogurt and control samples for non-fat iron fortified yogurt over one month of storage. The analysis of low -fat yogurt is still in progress.

**III. Sensory Analysis :** Quality of iron-fortified yogurt will be evaluated for appearance, texture, oxidizing off flavors (including oxidized, chemical and metallic flavors) by trained and untrained panelists during the second year of the project.

### **Impact of Research :**

We have shown that yogurt can be fortified with iron without affecting starter culture growth. Sensory evaluations are to be conducted during this coming year. If iron fortification does not adversely affect the flavor profile of yogurt, it would be possible to make iron fortified yogurts suitable for commercial manufacture.

### **Publications :**

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

Hekmat, S. and D. J. McMahon. 1993. Effects of iron fortification on chemical, physical and microbiological properties of yogurt. *J. Dairy Science* (In preparation).

Project Title: Extrusion processing of skim milk proteins

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

Hal Johnson, Professor, Food Science and Nutrition, Brigham Young Univ., Provo, Utah 84602

Funding: Western Dairy Foods Research Center

Objectives:

Extrusion has been shown to offer great product versatility by using a thermodynamically efficient method for the cooking, texturization and forming of raw materials with varied functional characteristics. American consumers are becoming increasingly aware of the nutritional importance of proteins, vitamins and minerals, and are avoiding foods containing excess calories and saturated fats. Therefore, new products may require the addition of selected sources of proteins such as skim milk proteins, production of which is an established segment of the dairy industry. However, research specifically designed to modify skim milk protein to lend itself more readily to inclusion in new food products is lacking.

This project will determine operating parameters for extruding skim milk proteins to alter their characteristics so that they will be more widely used in fabricated food products. This research is a cooperative effort between faculty and graduate students at Utah State University and Brigham Young University. A blend of corn starch and skim milk proteins will be extruded. The control variables will be:

- (I) Moisture Content
- (II) Protein Content
- (III) Lactose/Protein
- (IV) Feed Rate

The extrudates will be analyzed according to the following response variables:

- (I) Expansion Ratio
- (II) Bulk Density
- (III) Viscosity-cooked and uncooked
- (IV) Color Change
- (V) Water Absorption Index
- (VI) Shear Force
- (VII) Product Temperature

These tests will be done by USU researchers. In addition, USU will also do Scanning Electron Microscopy on the extrudates. B.Y.U. will test the modification of covalent and ion-covalent interactions. Since these two separate sets of data will be collected on the same extrudates, careful coevaluation will result

in a better understanding of extruder processing parameters, their effect on structural modification and the corresponding change in functionality.

## RESULTS

Based on preliminary runs the ranges for the control variables were determined. To get protein rich skim milk powder, skim milk was ultrafiltered to 13.67%, 17.07%, and 19.88% total solids concentration. The skim milk at various concentrations was analyzed for protein and lactose content. It was then dried to get protein rich skim milk powder. To generate the experimental design, surface methodology was used. A statistical software package called ECHIP was acquired for this purpose. Using ECHIP, a central composite rotatable design was generated. This design is the most accurate design for a quadratic model. The aim is to express the response variables in terms of the control variables via a quadratic model. Once this is done, a response surface will be generated and the optimum conditions for extrusion will be identified. To get the product temperature, some modification was done on the extruder. A space i.e. an extra segment of barrel was added between the screw and the die and a rigid thermocouple was inserted: This insured that the temperature being read was the product temperature, not the barrel temperature.

## IMPACT OF RESEARCH

This research will enable us to determine the optimum extrusion conditions. Knowledge of the optimum extrusion conditions will lead to the development of new protein rich extruded products. Also, great potential lies in extruding meat-dairy protein. The results of this study could open the doors for the use of skim milk proteins in various kinds of extruded products. In fact, we are confident that this research will provide the foundation for future work in this area.

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

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

Nabil Youssef, Dept. of Biology, USU.

Craig J. Oberg, Dept. of Microbiology, Weber State University

William McManus, Dept. of Biology, USU.

Robert Eife, Dept. of Nutrition and Food Sciences, USU.

**Funding:** Western Center for Dairy Protein Research & 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 (including those already conducted at Utah State University), our knowledge of why Mozzarella cheese stretches and melts is still very limited. Part of this information will be provided in a study being funded by the National Dairy Promotion and Research Board on the development of culture systems and the use of fat substitutes for the manufacture of low fat Mozzarella. This project focuses on the development of experimental techniques and equipment to study the microstructure of Mozzarella cheese under melting conditions using microscopy.

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 specific objectives of this project are to:

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

**Results:**

The use of Environmental Scanning Electron Microscopy (ESEM) was investigated for its application in dynamically studying the melting and stretching of Mozzarella

cheese. Although this equipment allows for the examination of cheese without the sample dehydration necessary when using traditional SEM methods it does not provide the information on the interior structure of cheese. At the magnification available using (ESEM) the surface structure of the melted cheese was not very well resolved. As an alternative, work was conducted to increase the resolution of our existing SEM procedures.

A metal impregnation technique using tannin-ferrocyanide-osmium tetroxide to impart thermal conductivity to samples destined for scanning electron microscopy examination was adapted for milk products. 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) as well as the factory specified resolution of microscope (2.2 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.

#### **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. This also has application for the study of many other types of dairy products.

#### **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 Structure. (Submitted for publication)

**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 Dairy Foods Research Center and Utah Agricultural Experimental Station

**Objectives:**

During the last decade, yogurt was used as a base-material to manufacture desirable cultured dairy product, namely yogurt-cheese or Labneh. Yogurt-cheese "Labneh" has been defined as the product made from ordinary yogurt after part of the water, lactose and salts are removed.

We are developing a new, non-traditional, high protein non-fat dairy fermented product fortified with different flavors, vegetables, or fruits. This product may also be used as a new dairy ingredient for fortification of foods. The results of the proposed project will fulfill the requirements for priority three of the National Dairy Board for FY 1993 to find new or non-traditional products.

The main objective of this research project is to develop a process to use yogurt-cheese manufactured from skim 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 the different types of consumers according to age, sex, health, and national origin.

1. Develop high protein, non-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:**

Some preliminary experiments have been carried out to determine the best conditions to produce the base ingredient of high protein, low fat fermented dairy products. Also, some flavors have been tried. We are trying to adapt suitable conditions to get a product with high acceptability according to data from a sensory evaluation being done.

**Significance:**

This project will develop a method to manufacture from ultrafiltered skim and/or low fat milk a yogurt-cheese to produce dairy product 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.

**Publications:**

None

**Project Title:** Development of a process for production of UF milk retentate

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

Donald J. McMahon, Professor, Dept. of Nutrition and Food Science, Utah State University

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

**Funding:** Western Dairy Foods Research Center and Utah Agricultural Experimental Station

**Objectives:**

Several methods are available for processing surplus milk to extend its shelf life. Skim milk powder has been the standard means for storing surplus milk solids. Recently a frozen concentrate has also been developed. The major problem associated with frozen milk concentrate is the gradual destabilization of casein micelles that occurs during storage. Successful ultra-high temperature (UHT) concentrated milk has not yet been achieved because of the gelation that occurs in such concentrates upon storage of room temperature.

Production of milk powder by evaporative concentration and drying is an established segment of the dairy industry. However, product quality considerations favor the use of membrane separation to concentrate milk rather than thermal processes. Extensive heating during evaporative concentration often causes product degradation, primarily through change of color and flavors, and high denaturation of protein

Milk powder with better functional and nutritional quality can be produced employing membrane separation such as ultrafiltration for concentrating milk.

The primary objective of this project is to develop a process for production of high protein UF milk retentate powder by:

1. Determining effects of heat treatment, pH and limited enzyme treatment of UF retentate on the chemical, physical and functional properties of its resultant spray dried, high protein, milk powder.
2. Determining effects of drying parameters, such as particle size, air temperature, solid concentration and foam spray, on properties of the retentate powder.
3. Evaluating product applications of the high protein milk powder.

## Results:

Raw skim milk retentate with 20% solids produced by UF was subjected to different heat treatments and pH adjustments prior to spray-drying. The heat treatments were 65°C for 30 min, 75°C for 28 s, and 85°C for 28 s. pH was adjusted to 6.4, 6.7, and 7.0. Retentate powders were analyzed for moisture, protein, lactose, fat, ash, titratable acidity, and pH. Physical property determinations included solubility index, dispersibility, viscosity, scorched particles, poured density, packed density, and water absorption isotherm. No interaction effects of heat treatment and pH adjustment were observed. pH adjustments affected ash content and solution viscosity. Heat treatment affected solubility and poured density. pH adjustments and heat treatment had only minor effects on the measured properties.

Skim milk retentate powder with modified functional properties was produced using UF and spray drying processes. Pre-treatments of different heat treatments and pH adjustments were applied prior to spray drying. The heat treatments were 65°C for 30 min, 75°C for 28 s, and 85°C for 28 s. pH was adjusted to 6.4, 6.7, and 7.0. The functional properties of the retentate powders were determined. pH adjustments significantly affected gel water-holding capacity, acid gel strength, emulsifying capacity and foaming capacity. Heat treatment also significantly affected gel water-holding capacity, acid gel strength, and emulsifying capacity, but not foaming capacity. The interaction of heat treatment and pH adjustment affected gel water-holding capacity, acid-gel strength and emulsifying capacity.

Results showed that by using UF to concentrate raw skim milk to 20% total solids, high protein retentate powders with >60% protein can be produced which is nearly double the protein concentration of conventionally produced skim milk powder. Also, functional properties can be modified by applying heat treatments and pH adjustments prior to spray drying.

## Significance:

Results showed that high protein retentate powders with modified functional properties could be produced by using UF to concentrate skim milk as well as applying heat treatments and pH adjustment prior to spray drying. One more advantage for retentate powders compared with other milk powders is that the retentate powders permit desirable usage in many food systems such as yogurt, cheese, ice cream and bakery products at more economic transportation costs. This powder is used now in our trials to develop a fermented dairy product which will be used as a dairy base ingredient to produce new dairy foods.

**Publications:**

1. Bond, S. A. and C. L. Hansen. 1991. Whippability of enzyme treated skim milk retentate powder. J. Dairy Sci. 74 (suppl. 1):95.

2. El-Samragy, Y. A., C. L. Hansen, and D. J. McMahon. 1992. Production of ultrafiltered skim milk retentate powder. 1. Composition and physical properties. J. Dairy Sci. 76:388-392.

3. El-Samragy, Y. A., C. L. Hansen, and D. J. McMahon. 1992. Production of ultrafiltered skim milk retentate powder. 2. Functional properties. J. Dairy Sci. In Process.

4. Hansen, C. L., E. A. Ahmed and Y. A. El-Samragy. 1993. Whipped topping from enzyme-hydrolyzed skim milk retentate. J. Dairy Sci. 79 (Suppl. 1):141.

**Project Title:** Controlling Age Gelation of UHT milk Concentrates

**Personnel:** Donald J. McMahon  
Conly L. Hansen  
Mohamed A. Mohamed  
Nutrition & Food Sciences Dept., Utah State University

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

**Objective:**

Age gelation of ultra-high-temperature (UHT) milk concentrates has hindered the commercial use of milk concentration as a means of lowering transportation cost. Composition of milk, severity of heat treatment, sequence of operation, homogenization, use of additives, total solids, and enzyme treatment has been shown to affect age gelation of UHT milk. The specific objective of this project is to study the effects of the process parameters of UHT heating on age gelation of milk concentrates.

**Results:**

Eight UHT runs (four direct and four indirect) were completed. Two pre-heat temperatures (75°C, 90°C) with two holding times (20 s, 120s), and two sterilization temperatures (138°C, 145°C) with two holding times (4,16 s) were used in this study. Samples were stored at two temperatures (15°C, 35°C) for eight months. Changes in viscosity, sediment depth, browning, and pH were monitored at four weeks interval.

Samples stored at 15°C showed an abrupt increase in viscosity resulting in complete gelation of all samples. Sedimentation occurred in all samples, regardless of the storage temperature, and the sediment particles were undispersible. The sedimentation depth increased with storage time and severity of heat treatment. Browning was observed in samples stored at 35°C and to some extent at 15°C for samples receiving higher heat treatment. The

rate of pH reduction was greater for samples stored at 35°C than those stored at 15°C. From these results we concluded the following:

- 1- The shelf life of UHT milk concentrates was longer for samples processed using the indirect method (indirect heat exchangers) and it was longer for samples held for longer time at higher temperature.
- 2- Sedimentation depth was greater for samples processed by the direct steam injection method and was directly related to the severity of heat treatment and length of holding time as well as the storage temperature.
- 3- Samples stored at 15°C showed an increase in viscosity compared to those stored at 35°C.
- 4- The rate of reduction in pH was greater for samples stored at 35°C.
- 5- Browning was intense for samples processed at higher temperature, for longer holding time and processed by the indirect method. The extent of browning was greater at higher storage temperature.

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

Based on the results obtained from this study, we recommend the indirect plate heat exchanger as a method for UHT processing with respect to age gelation of concentrated milk. Preheating the milk before sterilizing is a critical step for extending the shelf life of UHT milk concentrates. The longer the holding time during preheat treatment and during sterilization the longer the shelf life of the UHT milk concentrate. Storage temperatures in the range of 10-15°C is recommended for a longer shelf life.

#### **Publications:**

Mohamed M. A. and D. J. McMahon. 1993. Influence of ultra-high temperature process parameters on age gelation of milk concentrate. J. Dairy Sci. 76 (Supplement 1):104.

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

**Personnel:** Donald J. McMahon

M. Christopher Alleyne

Nutrition & Food Sciences Dept., Utah State University

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

Utah Agricultural Experimental Station

**Objectives:**

1. To determine the influence of lactose concentration of milk concentrates on age gelation.
2. To determine the role of whey proteins (especially  $\beta$ -lactoglobulin) in the mechanism of age gelation of UHT sterilized milk concentrates.
3. To monitor changes in casein micelle structure that occur during storage of UHT sterilized milk concentrates and relate this to their stability

**Results:**

**Objective 1.** This has been completed.

**Objective 2 & 3.**

Using antibodies to  $\beta$ -lactoglobulin (J.J. Stastny, University of Illinois College of Medicine), Goat anti-mouse IgG conjugated to 10 nm gold (Ted Pella Inc.) and other electron microscopy and immunogold labeling materials a protocol for immunolabeling of milk samples was established and this will be applied to the other 5 milk proteins to determine their positions in fresh milk through UHT processing and storage until gelation. A tentative mechanism for concentrated UHT milk age gelation has been 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 0.5 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 properly the filled capsules. 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 microencapsulation but shows agar fibres in the micrographs of the milk samples. This is particularly undesirable when investigating, for example, intermicellar strands of gelled UHT milk concentrates. Microcube encapsulation produces superior quality images of the fluid food structure.

### **Abstract 2.**

Trichloroacetic acid precipitated milk whey protein from direct ultra-high temperature processed milk retentate (ultrafiltration concentrated 3x by volume reduction) was used to establish a protocol for aldehyde fixation and immunogold labeling of  $\beta$ -lactoglobulin in milk samples. Microcube encapsulation was the prefixation method for all samples. ELISA was used to investigate antigen degradation caused by aldehyde. Antigenicity of the samples was reduced by both aldehyde fixatives but paraformaldehyde was 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 0.5 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 preserved antigenicity while providing adequate fixation of the protein. The paraformaldehyde fixation resulted in better sections at labeling. LR White resin polymerized at 50°C was satisfactory for the embedding of samples. Teleosteam fish gelatin (0.1%) with normal goat serum (0.1%) in 20mM tris buffer without bovine serum albumin proved to be an



adequate blocking agent. The protocol was then used on gelled UHT and fresh UHT milk retentates to localize  $\beta$ -lactoglobulin.

### **Abstract 3**

$\beta$ -Lactoglobulin complexed with other milk proteins can be identified in dairy foods using immunolocalization techniques. Immunolocalization with embedding at 50°C in LR White resin was used to identify complexed  $\beta$ -lactoglobulin in eleven dairy products. The effect of the manufacturing process on the location and relative abundance of this whey protein was investigated. Heating temperature, duration of heating and pH during manufacturing affected the production of  $\beta$ -lactoglobulin complexes with other milk proteins. More stable  $\beta$ -lactoglobulin complexes were produced around an optimum temperature and low pH.  $\beta$ -Lactoglobulin complexed with caseins was more stable than  $\beta$ -lactoglobulin complexed with other whey proteins. The ripening process of cheese seemed to destabilize or remove  $\beta$ -lactoglobulin complex from the product. This immunolocalization technique can be used to identify  $\beta$ -lactoglobulin complex in other food systems and should prove useful in investigating fundamental manufacturing effects on the fate of this protein in foods.

### **Impact of Research:**

A mechanism of age gelation, elucidated through immunolocalization techniques and transmission electron microscopy, may be proposed.

### **Publications:**

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

Alleyne, M. C., W. McManus and D. J. McMahon. 1993. Comparison of antigenicity of  $\beta$ -lactoglobulin in UHT milk retentate and whey different modes of aldehyde fixation. *Food structure (Approved for Submission)*

Alleyne, M. C., W. McManus and D. J. McMahon. 1993. Immunolocalization of  $\beta$ -lactoglobulin in processed milk, yogurt and cheese samples. *Food structure (Approved for Submission)*

## WCDPRT PROGRESS REPORT

June 7, 1993

**Project Title:** Cloning the Nisin and Other Genes of Lactic Streptococci (lactococci) into *Leuconostoc* Species and Amplification of Nisin Production.

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

### Objectives:

1. To produce and characterize lactose positive *Leuconostoc* transconjugants obtained by conjugal matings between *Lactococcus lactis* and *Leuconostoc* spp.
2. To develop transformation and gene cloning systems in *Leuconostoc*.
3. To introduce into *Leuconostoc*, plasmid-coded-protease genes from lactic streptococci.
4. To use the genetically constructed fast acid-producing *Leuconostoc* to produce different fermented dairy products such as cultured buttermilk, cottage and Gouda cheese.
5. To conjugally transfer plasmid-coded nisin genes from *Lactococcus lactis* to *Leuconostoc*.
6. To amplify nisin production by gene cloning techniques.
7. To use nisin producing *Leuconostoc* in Swiss cheese manufacture to inhibit gas producing anaerobic spore formers such as *Clostridium tyrobutyricum*.
8. To study the inhibition of *L. monocytogenes* by nisin and to use genetic engineering to maximize its useful application.

## Results:

Results reported in this report are only those that have been obtained since the last progress report dated May 29, 1992. Using the transformation system developed and reported on in the last report and also published in the Journal of Dairy Science (74;1454, 1991), efforts were concentrated on introduction of lactococcal genes into *Leuconostoc*. This was facilitated by the isolation and characterization of a cryptic stable plasmid from *Enterococcus faecium* 226. It is a 3.8 Kb stable vector with 18 unique restriction sites. Sequence analysis of the minimal replicon identified a possible origin of replication and putative replication protein. Analysis showed that the vector was not of the single-stranded rolling circle replication type common to Gram positive bacteria. It was also found to be an extremely stable replicon with limited homology to the well-known lactococcal plasmid pCI305. Sequence analysis of the origin detected one open reading frame of 822 base pairs capable of encoding a 32 Kdalton protein corresponding to the results of an in vivo transcription/translation reaction. No detectable single-stranded intermediates were found for the replicon, suggesting that the cryptic plasmid, designated pMBB1, may be included in the same family as the widely used plasmid pCI305. Using the pMBB1, a small stably maintained vector containing a large multiple cloning region was constructed. This vector was successfully used in electroporating the  $\beta$ -galactosidase gene from *Streptococcus thermophilus* into *Leuconostoc* strains. High levels of the enzyme were detected in cell-free extracts of several *Leuconostoc* species which had either no enzyme or very low levels. This is the first recorded instance of transfer of genes from lactococci into *Leuconostoc* and while the  $\beta$ -galactosidase containing *Leuconostoc* were unable to ferment lactose, we now are coming more close to realizing this possibility. It is likely that the permease gene was not "transformed" along with structural  $\beta$ -galactosidase.

In other work related to the transfer of genes from lactococci to *Leuconostoc*, two *Lactococcus lactis* strains (LM2301 and LM2306) were found useful for insertional activation studies with the streptococcal transposon Tn919. In contrast to a previous report concerning *Lactococcus lactis* MG1363, we have found that Tn919 inserts into the chromosome of both LM 2301 and LM2306 in a multiple and random manner. This is significant in that it reports two possible candidates for insertional activation studies with Tn919 in *L. lactis*, which previously were

thought to be limited. Insertional activation of genetic information into the bacterial chromosome is a highly useful method to follow gene transfer and use of this transposon with the indicated strains will be useful in the future in following the introduction of lactococcal genes into *Leuconostoc*. Being able to do this will greatly improve the utility of *Leuconostoc* bacteria.

In the process of doing these genetic studies on the *Leuconostoc*, we were frustrated by the lack of a thorough study of the many *Leuconostoc* organisms that are in our culture collection. This was a hindrance to our work and so we embarked on a more thorough characterization of these bacteria in order to find strains that were suitable candidates for continued genetic researches. From our collection of over 100 strains, we did a thorough taxonomic study with the objective of determining for certain which strains were *Leuconostoc cremoris*. These are the ones that we could most easily identify to species and ones we are confident will prove useful in manufacturing dairy fermented products. About 20 strains of *Leuconostoc cremoris* have been thoroughly characterized to insure their identity and determine the variety of biochemical properties which they possess and these strains also are being analyzed for their ability to produce diacetyl in association with lactococci. They also are being analyzed for bacteriocin-producing capabilities, since strains producing these inhibitory substances would not be good candidates for use in fermented dairy products since they likely would inhibit the lactococcal strains with which they were grown. Several *Leuconostoc* have been found which are inhibitory for lactococci and this emphasizes again the need to be able to construct *Leuconostoc* strains with protease and  $\beta$ -galactosidase activities so that they can be used as single cultures in the manufacture of fermented dairy products.

Objectives 5 through 8 of this research project were studied by Dr. Kondo and Dr., Broadbent at Utah State University. Their most recent progress has been communicated in the form of an abstract of a paper to be presented at the 1993 meetings of the American Dairy Science Association at the University of Maryland, June 13 - 16. The abstract of that paper follows:

"Conjugation was used to transfer genes associated with nisin production from nisin- and sucrose-positive ( $Nip^+Suc^+$ ) *Lactococcus lactis* subsp. *lactis* donors to sucrose-negative ( $Suc^-$ ) *Streptococcus salivarius* subsp. *thermophilus*

recipients. Streptococcal recipients were constructed by electro-transformation with the erythromycin-resistance plasmid pGK13, or by conjugal introduction of the enterococcal plasmid pAM $\beta$ 1. Matings between lactococcal Nip<sup>+</sup>Suc<sup>+</sup> donors and streptococcal recipients that contained pAM $\beta$ 1 yielded Suc<sup>+</sup> transconjugants that were immune to 7 mg per ml of nisin but which did not produce the bacteriocin. Genetic and phenotypic analysis of these transconjugants demonstrated that they had acquired the nisin structural gene, *nisA*, as well as other genes encoded by the lactococcal nisin-sucrose conjugative transposon, Tn5301. Finally, transconjugants were not detected in matings with recipients that contained pGK13, which suggested that pAM $\beta$ 1 may have facilitated intergeneric transfer of nisin genes."

### Impact of Research

Having demonstrated ability to transfer genes from lactococci into *Leuconostoc*, it may be expected that future studies with this system will enable us to construct strains which will be improved for use in dairy fermentations. *Leuconostoc* able to ferment lactose and degrade milk protein will be highly useful in the manufacture of more uniform dairy products and also for use in the production of new specialty dairy products.

The work with nisin had the ultimate objective of being able to construct super nisin producing strains for use in food preservation. While transfer of the nisin genes has been accomplished, continued research is necessary in order to clone those genes into stable vectors which can be used to amplify the nisin production. Such an accomplishment would be highly significant in terms of producing large amounts of nisin for use by the dairy industry.

## **Publications:**

Broadbent, J. R., J. K. Kondo and W. E. Sandine. Intergeneric transfer and Expression of Genes Associated with Nisin Production. *Journal of Dairy Science*, 76(supplement 1):122, 1993.

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**Project Title:** Cloning the Nisin and other Genes from *Lactococcus* into *Leuconostoc* Species and Amplification of Nisin Production.

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UTAH AGRICULTURAL EXPERIMENT STATION  
NATIONAL SCIENCE FOUNDATION  
USDA-ARS NATIONAL NEEDS FELLOWSHIP PROGRAM

**Objectives:**

1. To develop transformation and gene cloning systems in *Leuconostoc*.
2. To introduce into *Leuconostoc*, plasmid-coded protease genes from lactococci (e.g. *Lactococcus lactis* subsp. *cremoris* Wg2).
3. To study conjugal transfer of nisin production and introduce these genes into species of lactic acid bacteria.
4. To amplify nisin production by genetic manipulation techniques.
5. To study the inhibition of *Listeria monocytogenes* by nisin and to use genetic engineering techniques to maximize its useful application.

**Results:**

Initial studies at OSU demonstrated that nisin inhibited several strains of *Listeria monocytogenes*. Sensitivity among *L. monocytogenes* strains varied widely, with MICs ranging from 740 to  $10^5$  IU/ml (Benkerroum and Sandine, 1988). Subsequent investigations at OSU focused on the genetic manipulation of dairy *Leuconostoc* spp. and the development of gene cloning systems in this important genus (Wycoff, 1992).

Conditions that allowed electro-transformation of *L. mesenteroides* subsp. *dextranicum*, *L. mesenteroides* subsp. *cremoris* and *L. lactis* at frequencies up to  $2 \times 10^6$  transformants per  $\mu\text{g}$  of DNA were determined using several plasmid vectors (Wycoff et al., 1991). This result was crucial to the study because it opened the door to

the application of recombinant DNA technology in *Leuconostoc* spp. In related work on gene cloning, we discovered a 3.8 kb enterococcal plasmid, pMBB1, that replicated very stably in *Leuconostoc* spp. and other lactic acid bacteria. Because pMBB1 had unusually high stability (for plasmid DNA) in lactic acid bacteria, we felt that it would be a useful chassis for the construction of a stable cloning vector for *Leuconostoc* spp. As a result, pMBB1 was completely sequenced to identify open reading frames (ORFs) and all restriction enzyme sites (Wycoff et al., 1993). In vitro transcription/translation assays indicated that pMBB1 encoded a single 32 kDa protein and this observation was supported by the DNA sequence analysis. Our *Leuconostoc* cloning vector, pHW800, was then constructed from the pMBB1 replicon by adding a gene for chloramphenicol resistance to facilitate selection and a multiple cloning site to facilitate the insertion of foreign DNA.

Some analyses of electroporation parameters were also performed at USU to investigate the effects of electroporation parameters on other bacteria, such as lactobacilli and lactococci. This work yielded a protocol that provided electro-transformation of *Lactobacillus* spp. and lactococci at frequencies around  $10^3$  transformants per  $\mu\text{g}$  DNA (Chou, 1992). Although the transformation frequency was not particularly high, the protocol worked with several genera of lactic acid bacteria including *Lactobacillus*, *Lactococcus*, *Streptococcus* and *Leuconostoc*. The availability of an electroporation protocol that provides reasonable transformation frequencies with several genera of lactic acid bacteria should be valuable for the rapid distribution of cloned DNA among lactic acid bacteria.

The development of transformation and gene cloning systems for *Leuconostoc* spp. at OSU provided the tools needed to try and construct fast acid-producing *Leuconostoc* using recombinant DNA technology. Fast acid production in milk requires the ability to rapidly utilize lactose ( $\text{Lac}^+$ ) and milk proteins ( $\text{Prt}^+$ ). To introduce  $\text{Lac}^+$ , a 5 kb fragment of DNA that encoded genes for lactose transport and  $\beta$ -galactosidase in *Streptococcus thermophilus* was isolated using the polymerase chain reaction, cloned into the vector pGL3, and electro-transformed into *Leuconostoc* spp. Analysis of *Leuconostoc* transformants revealed that they produced very high levels of  $\beta$ -galactosidase but were apparently unable to transport lactose. Further analysis of the clone in *E. coli* and lactococcal hosts indicated that the lactose permease gene was not expressed in these bacteria. Further experiments are required to determine whether a functional lactose permease gene can be expressed in *Leuconostoc* spp.

As reflected by objective 2, we sought to improve the ability of *Leuconostoc* spp. to use milk protein by introducing genes for the lactococcal extracellular proteinase. to



achieve this, a 4.7 kb fragment of pJK550 which carried genes for the extracellular proteinase of *Lactococcus lactis* subsp. *lactis* C20, was cloned in pGK13. Analysis of the clone in a Prt<sup>-</sup> lactococcal host showed that the proteinase gene construct was functional. The recombinant plasmid was purified, electro-transformed into *Leuconostoc* spp., and plasmid analysis was performed on transformants. Surprisingly, all of the *Leuconostoc* transformants we examined contained a plasmid that has suffered deletions or insertions in the portion which encoded the proteinase genes. Because our previous work had demonstrated that pGK13 could be transformed into these *Leuconostoc* without any of these difficulties (Wycoff et al., 1991), and that the proteinase genes it carried were functional in lactococci, we believe that the instability problem was directly related to the expression of the lactococcal proteinase genes in *Leuconostoc*. These observations indicated that expression of genes for the lactococcal extracellular proteinase was probably lethal to *Leuconostoc* spp. and so this enzyme could not be used to improve the ability of *Leuconostoc* to cleave milk proteins.

Research related to gene transfer at OSU also investigated the use of insertional inactivation of lactococcal genes using the streptococcal transposon Tn919. Those studies discovered that the transposon inserts multiple copies of itself into the chromosomes of *Lc. lactis* subsp. *lactis* LM2301 and LM2306 in a random manner (Barnes et al., 1993). This result is significant because insertional inactivation of genes is a very useful way to follow gene transfer and will be useful in the future to follow lactococcal genes introduced into *Leuconostoc* spp.

Research at USU focused on the genetics of nisin production in *Lc. lactis* subsp. *lactis*. Our initial studies sought to determine whether genes for nisin and sucrose were encoded by plasmid or chromosomal DNA. Results from plasmid curing, conjugation and DNA-DNA hybridizations to an oligonucleotide probe for the nisin structural gene showed that this gene was chromosomally encoded in *Lc. lactis* subsp. *lactis* strains 11454, 7962, DL16 and 496 (Broadbent et al., 1993a).

We also studied conjugation of the nisin-sucrose phenotype (Nip<sup>+</sup>Suc<sup>+</sup>) in a lactococcal model system to develop methodology for transferring these genes to other lactic acid bacteria. These experiments led to an improved conjugation technique which facilitated transfer of nisin and sucrose genes into strains of *Lc. lactis* subsp. *cremoris* and *Streptococcus thermophilus*. Transfer of Nip<sup>+</sup>Suc<sup>+</sup> from the donor strain, *Lc. lactis* subsp. *lactis* 11454, to *L. lactis* subsp. *cremoris* recipient strains occurred at frequencies which ranged from 10<sup>-7</sup> to 10<sup>-8</sup> per donor CFU. Analysis of *L. lactis* subsp. *cremoris* Nip<sup>+</sup>Suc<sup>+</sup> transconjugants indicated that they retained the parental fast acid-producing capability in milk (Broadbent and Kondo, 1991). Surprisingly, when nisin production in

these transconjugants was measured, we found that some transconjugants produced up to three times as much nisin as the donor strain 11454. DNA-DNA hybridizations between the oligonucleotide nisin gene probe and genomic DNA isolated from one of the *Lc. lactis* subsp. *cremoris* transconjugants demonstrated that the nisin structural gene, *nisA*, could exist in more than one copy in some *Lc. lactis* subsp. *cremoris* transconjugants. Thus, elevated nisin production in transconjugants may be due to a gene dosage effect. Similar observations have been made in Nip<sup>+</sup>Suc<sup>+</sup> transconjugants of *Lc. lactis* subsp. *lactis* (W.M. DeVos, pers. comm.). These observations indicate that conjugation can be used to amplify nisin production levels in strains of *Lc. lactis*.

Matings between *Lc. lactis* subsp. *lactis* 11454 and *S. thermophilus* recipients yielded transconjugants that expressed Suc<sup>+</sup> and nisin immunity (Nis<sup>i</sup>) but did not produce nisin (Broadbent et al., 1993b). The frequency of nisin gene transfer to *S. thermophilus* recipients was approximately  $2 \times 10^{-9}$  transconjugants per donor CFU. DNA-DNA hybridizations between the oligo-*nisA* probe and genomic DNA isolated from streptococcal transconjugants demonstrated that the nisin structural gene had been transferred to these cells. Our conjugation studies showed that it is feasible to construct nisin-producing *Lc. lactis* subsp. *cremoris* and nisin-immune *S. thermophilus* strains for application in nisin-producing dairy starter systems. Unfortunately, similar experiments with recipients of *Leuconostoc* spp. or *Lactobacillus helveticus* failed to detect nisin-sucrose conjugation.

In an effort to overcome the barriers we encountered with conjugation, we investigated the use of protoplast fusion to effect gene transfer between *L. mesenteroides* subsp. *dextranicum* 181 and *Lc. lactis* subsp. *lactis*. Although conditions for cell wall removal and regeneration in *Leuconostoc* and lactococci were established, efforts to detect gene transfer by protoplast fusion were not successful.

Additional studies of conjugation were performed to investigate the relationship between donor cell aggregation and high-frequency transfer (Hf-T) of lactose plasmids in lactococci. Results from those studies showed that cell clumping and Hf-T in lactococci was quite similar to hemolysin plasmid exchange in *Enterococcus faecalis*. Our study also identified a novel 125 kDa protein that is likely essential for cell clumping and Hf-T of lactose utilization in transconjugants of *Lc. lactis* subsp. *lactis* ML3 (Wang, 1992, Wang and Kondo, 1991).

Finally, a 10 kb fragment that contained the nisin gene and downstream DNA was cloned and transformed into *Lc. lactis* subsp. *lactis* LM0230. Phenotypic analysis of electro-transformants did not reveal expression of nisin production or immunity, sucrose

utilization, or reduced phage sensitivity. To determine whether we had isolated all of the operon that includes the nisin structural gene, restriction analysis and DNA sequencing of the fragment were performed. Those results demonstrated that our fragment contained the *nisA*, *nisB*, *nisT* and *nisC* gene sequences reported by others (Engelke et al., Appl. Environ. Microbiol. 58:3730, 1992). DNA sequence analysis of 2.5 kb of downstream DNA not previously characterized revealed two ORFs (ORF1 and ORF2) which encoded proteins of 245 and 505 amino acids. Sequence data also showed that ORF2 had been truncated on the 3' end by cloning which indicated that our fragment did not encode the entire nisin operon.

A database search for protein homologies to ORF1 and ORF2 found no significant homology to ORF1 but good homology between ORF2 and the amino acid sequences of subtilisins and EpiP. EpiP is a protein involved in the biosynthesis of the lantibiotic epidermin and is thought to cleave the signal sequence from pro-epidermin to generate mature epidermin (Schnell et al., Eur. J. Biochem. 204:57, 1992). As a result, ORF2 was designated *nisP*. Recently, van der Meer et al. (J. Bacteriol. 175:2578, 1993) reported that the intact NisP protein is 682 amino acids in length and that its' gene is followed by at least one additional ORF, *nisR*.. Their report also mentioned the ORF we had called ORF1 and designated it *nisl* because of a suspected role in nisin immunity. In summary, the worldwide attention given to nisin biosynthesis has dramatically improved our understanding of this process during the past 5 years. Heterologous expression of nisin production in *Lactococcus* from cloned DNA appears imminent and, once realized, will create opportunities to study the expression of these genes in other lactic acid bacteria, including *Leuconostoc* spp.

#### **Impact of Research - Nisin:**

Nisin, a peptide antibiotic produced by some strains of *Lc. lactis* subsp. *lactis*, is an effective inhibitor of Gram-positive bacteria. The antibiotic has been approved for use as a food preservative in over forty-five countries, including the United States. For years, investigators have been interested in transferring the nisin genes into other organisms used to manufacture fermented foods to enhance the shelf life of these products. Conjugation, a natural gene transfer process, may be useful to achieve this goal. Bacterial strains that are developed via conjugation contain genes that only come from other safe, food-grade lactic acid bacteria. Consequently, conjugally improved strains may face fewer FDA restrictions, with respect to industrial application, than strains which are improved through recombinant DNA technology. Our studies of conjugation yielded methodology which allowed us to transfer these genes into strains

of *Lc. lactis* subsp. *cremoris* and *S. thermophilus*. Even greater distribution of these genes should be possible through the use of recombinant DNA technology, although the regulatory barriers these strains may face is presently unclear. Our research provided basic and applied information on the expression of nisin genes in lactic acid bacteria, including valuable insight into the potential limitations these constructs may encounter. We can foresee many applications for nisin-producing or nisin immune lactic organisms among dairy, food, and agricultural fermentations, to enhance the safety and shelf-life of foods.

### **Construction of rapid acid-producing *Leuconostoc*.**

*Leuconostoc* spp. are slow acid-producers in milk and thus are unable to produce appreciable amounts of the important flavor compound, diacetyl, in pure milk cultures. This shortcoming has been attributed to their limited ability to metabolize milk proteins and lactose. If the growth of *Leuconostoc* spp. is slow or inhibited, the fermented product lacks proper flavor. By increasing the levels of proteolysis and lactose utilization, through gene transfer from lactococci to *Leuconostoc* spp., it may be possible to reduce or eliminate flavor defects in milks fermented with *Leuconostoc* spp. Genetically modified *Leuconostoc* spp. may also have application for the manufacture of specialty cheeses similar to varieties now imported, or the development of novel dairy products. Our research has generated the transformation and gene cloning systems which were required for the application of recombinant DNA technology in *Leuconostoc* spp. It also provided valuable insight into some of the unpredictable difficulties that face heterologous gene expression in this important genus, and the need for fundamental research into the physiology of these organisms.

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## WCDPRT PROGRESS REPORT

June 10, 1993

**Project Title:** Growth of Bifidobacteria in Milk: Association with *Streptococcus thermophilus* and *Lactobacillus* species as measured by genetic and enzyme probes.

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

### Objectives:

1. Development of an antibody against fructose-6-phosphate-phosphoketolase (F6PPK). Fructose-6-phosphate-phosphoketolase is an enzyme unique to bifidobacteria. Antibody production necessitates purification of the enzyme in quantities sufficient for injection into rabbits. With the antibody in hand, Western blots of colony lifts of plated cultures from fermented dairy products can be performed.
2. Development of a genomic probe against the gene for fructose-6-phosphate-phosphoketolase. Achievement of a homologous genomic probe necessitates the cloning of the gene for fructose-6-phosphate-phosphoketolase. *E. coli* cells containing the cloned gene can be identified from a library of cloned sequences by screening the library with the antifruuctose-6-phosphate-phosphoketolase antibody.

## Results:

In the last report we described the purification to homogeneity of the enzyme fructose-6-phosphate-phosphoketolase, a protein exclusively found in bifidobacteria. Since that time experiments to optimize growth conditions for the bifidobacteria to maximize enzyme production and stability have been conducted and the best medium found to date for growing the organisms for enzyme extraction has been determined to be half strength PMN broth. Half strength PMN broth contains, per liter; 25 g of Peptonized Milk Nutrient obtained from Sheffield Products, a division of Quest International, in Norwich N.Y., 20 g of primaton meat extract also obtained from Sheffield Products, 1.0 g of yeast extract, 5 g of lactose and 1.0 ml of Tween 80.

Originally it was intended to use the rabbit prepared antisera for developing of an enzyme linked immunosorbant assay (ELISA) but it since has been decided to use monoclonal antibody. Oregon State University has an operational monoclonal antibody facility and leukocytes from immunized responsive mice have been collected and fused with a mouse myeloma cells. Residual spleen cells have been frozen for subsequent fusions as well. At the present time spent medium from hybridoma cultures are being tested for the presence of antibody that binds to the fructose-6-phosphate-phosphoketolase. Positive cells will be grown in expanded cultures and frozen for subsequent use in developing the ELISA assay. Prior to preparing the monoclonal antibody, a nitrocellulose colony lift procedure was developed for establishing the colony matrix upon which the ELISA tests will be conducted.

The presently used purification procedure of the fructose-6-phosphate-phosphoketolase involves essentially four main steps: 1) Bead beating for extract production; 2) protamine sulfate precipitation for removal of the nucleic acid; 3) Sephacryl S-300 gel filtration; 4) Mono-Q anion exchange chromatography using fast pressure liquid chromatography. Beginning with crude extract that has about 5 units of activity per mg of protein, the purified material has a specific activity of well over 100 units per mg of protein. Percentage recovery of activity units of enzyme in the crude extract is arbitrarily established at 100 and in the purified material approximately 40% of those units are recovered.



Experiments are now in progress to characterize the enzyme thoroughly with respect to optimum temperature, optimum pH, kinetic properties, nature of active site as revealed by inhibitor studies and stability characteristics. Also in progress is construction of a genomic library from *Bifidobacterium brevis* from which the gene for F6PPK will be cloned. With the cloned gene in hand, the sequence for F6PPK can be obtained and a gene probe based on those sequences can be constructed. The gene probe will be used to hybridize to colony lifts of plated bifidobacterial-containing yogurt cultures to provide an alternative method of quantitating viable bifidobacterial cells in dairy products.

### **Impact of Research:**

Sweet Acidophilus milk has represented a significant product for the dairy industry in terms of volume consumed. The same may be said of yogurt, some of which now reveal on the label the presence of *Lactobacillus acidophilus* and even bifidobacteria. Bifidobacteria are promoted in Japan and also in Europe as bacteria which produce unique health benefits including elevation of immunocompetence, and reduction in the incidence of colon tumors. Data in the literature from well controlled scientific experiments support these conclusions (see review by Sanders entitled "The effect of lactic cultures on human health", Advances in Food Nutrition Research, In Press). Furthermore, research sponsored by the California Dairy Foundation is now underway to develop the next generation dairy product of this type. It will contain *Lactobacillus acidophilus*, *Bifidobacterium* species, and *Streptococcus thermophilus*. This product will offer advantages to those who suffer from lactose malabsorption and deliver strains of lactobacilli and bifidobacteria which will adhere to the intestinal tract. To produce uniformly high quality products containing bifidobacteria requires a simple method of accurately enumerating the number of those types of bacteria present. While many selective media have been described for use in enumerating bifidobacteria, each suffers from limitations such as lack of selectivity and suitability, only for certain strains. Therefore, the ELISA and gene probes are justified on the basis of providing a sensitive and accurate method for determining exact numbers of bifidobacteria present in fermented products containing these bacteria. This will allow establishment of minimum standards of viable bifidobacteria as now exists for numbers of lactobacilli in Sweet Acidophilus milk ( $2 \times 10^6$  per ml). This way the customer will be assured of

consuming products which contain an appropriate minimum number of bifidobacteria at the time of consumption. Use of the selective method based on the ELISA technique also will allow future studies to examine strain and species variability among the bifidobacteria to select those that are the most desirable for use in fermented dairy products. There is little doubt that the future will see more and more use of bifidobacteria in fermented products and the technologies offered by this research will facilitate that.

**Publications:**

None.

**Project Title:**      **Characterization of Milk Proteolysis by Lactococcal Starter Culture Strains Using Amino Acid Analysis**

**Personnel:**        Rodney J. Brown  
                         Christina Beer  
                         Nutrition & Food Sciences, Dept., Utah State University

**Funding:**            WESTERN DAIRY FOODS RESEARCH CENTER

**Objectives:**

1.    To genetically construct lactococcal strains with different proteolytic (PrtP and PrtT) and lactose utilizing capabilities.
2.    To use amino acid analysis to study the interactions of milk proteolysis genes (PrtP and PrtT) of the genetically constructed strains.
3.    To characterize growth and acid production of the genetically constructed strains and determine the effects that maintenance of plasmids have on growth and acid production.
4.    To examine the effect of different expression levels of proteinase genes (PrtP and PrtT) to try and improve flavor and texture characteristics in cheese, and examine the potential of these results for application to accelerated cheese ripening.

**Results:**

**Objective 1:**

    This part of the research has been completed.

**Objective 2:**

    Amino acid analysis has been done on the strains when grown in whole pasteurized milk. The proteolysis of individual caseins is in progress and will be completed at the end of August.

**Objective 3:**

    Characterization of growth and acid production has been completed. The characterization of plasmid maintenance will begin in August.

**Objective 4:**

    This objective has not been completed yet.

### **Impact of Research:**

Proteolysis from bacterial starter cultures play a significant role in the physical and organoleptic properties of cheese and other fermented dairy products. Improper proteolysis can result in a wide number of defects, including bitterness, texture, and body problems. We have ways to measure gross proteolysis but are very limited in techniques to profile or characterize proteolysis for individual bacterial strains. Understanding of the specific action of proteinases and peptidases upon specific milk proteins and a correlation of that activity to specific physical and organoleptic properties would be of great value to the dairy industry. This project has the potential to greatly enhance product quality, allow for the production of products with enhanced properties, and even allow the development of new products by using bacterial strains with different proteolytic abilities. This method would also be very valuable in identifying and characterizing newly developed strains for biotechnological endeavors.

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

**Personnel:** Bruce Geller, Assistant Professor, Department of Microbiology, Oregon State University.

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

**Objectives:**

1. Make *in vitro* mutations in the cloned gene required for phage infection.
2. Insert the mutated copies of *pip* into the chromosome and exchange it with the wild-type gene.
3. Test the different mutants for viability, phage resistance, and growth characteristics favorable to cheese-making.

**Results:**

We have screened phage m13-resistant mutants of *Lactococcus lactis* subsp. *lactis* ML3, and found some that are complemented by pBG1. This confirms that *pip* is required for phage infections in more than one strain of *Lactococcus lactis* subsp. *lactis*. In addition, the phage m13-resistant mutants were resistant to 11 other phages, but were complemented to the phage-sensitive phenotype when transformed with *pip*. This suggests that *pip* may be required for many, and perhaps most phage infections in strain C2 and ML3. We are currently screening phage C2-resistant mutants in *Lactococcus lactis* subsp. *cremoris* KH to determine if *pip* is required in other subspecies.

We have begun to sequence the mutated copy of *pip* from the spontaneous phage c2-resistant strain of *Lactococcus lactis* subsp. *lactis* C@, that we named RMC2/4. We have determined that *pip* is fully contained on a unique, 12 kb XbaI fragment of the chromosome from *Lactococcus lactis* subsp. *lactis* C2. Knowing this we made a genomic library of XbaI digested chromosomal DNA from RMC2/4. We are in the process of screening *E coli* clones for mutated *pip*, using colony hybridization with a labeled wild-type *pip* as a probe.

We have initiated studies to determine if *pip* is required for viability. To do this we will exchange, by homologous recombination, the wild-type chromosomal copy of *pip* for a Tn5-mutated copy of *pip*. The Tn5-mutated copy of *pip* was constructed in the course of our investigation of the wild-type *pip* (Geller *et al.* 1993, J. Bacteriol., in press). We experienced a technical difficulty in sub-cloning Tn5-*pip* into an appropriate integration vector, in that the XbaI fragment containing Tn5-*pip* in pSA3 was exactly the same size as XbaI-restricted pSA3. Therefore, we could not separate the two fragments by electrophoresis. We solved that problem by finding a restriction site (KpnI) on pSA3 that is not present on the XbaI fragment of Tn5-*pip* from pSA3. This allowed us to reduce the size of the XbaI fragment of pSA3, separate the Tn5-*pip* XbaI fragment by electrophoresis, and sub-clone the latter into a modified pACYC184. We found it necessary to modify pACYC184 by replacing the tetracycline resistance gene with the erythromycin resistance gene (from pSA3), in order to have a selectable

marker in *Lactococcus*. Currently we are attempting to select chromosomal integrants of the Tn5-*pip*.

**Impact of research:**

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.

This research would create new strains of lactic bacteria for starter cultures that are more phage-resistant than currently available. This strains 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.

**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  $\beta$ -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  $\beta$ -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:**

Project objectives 1 through 3 were completed during the first and second years of the project. The adsorption kinetics exhibited by  $\alpha$ -lactalbumin ( $\alpha$ -lac),  $\beta$ -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  $\beta$ -lactoglobulin ( $\beta$ -lg) adsorption kinetics on silicon surfaces varying in wettability. With regard to  $\beta$ -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,  $\alpha$ -lac,  $\beta$ -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  $\alpha$ -lac,  $\beta$ -casein,  $\beta$ -lg and BSA interfacial behavior at air-water interfaces, and  $\alpha$ -lac,  $\beta$ -lg and BSA interfacial behavior at solid-water interfaces.  $\beta$ -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  $\alpha$ -lac and BSA behaved in a manner more similar to that of  $\beta$ -lg at each surface; however, the difference in  $\alpha$ -lac adsorption to hydrophilic and hydrophobic surfaces was quite large, with  $\alpha$ -lac adsorbing to a much greater extent on hydrophobic surfaces.  $\alpha$ -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  $\alpha$ -lac in the same time period. 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 Image analysis was used for visualizing and enumerating bacterial cells adhered to surfaces. Initial results clearly indicated significant differences in the numbers of adhered cells to surfaces with different adsorbed proteins. Most dramatic were BSA treated surfaces which had 4-5 fold less adhered bacteria as compared to the non-globular proteins.

### Impact of Research:

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:

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. Surface hydrophobic influences on  $\beta$ -lactoglobulin adsorption kinetics. *J. Colloid Interface Sci.*, 154:337

Suttiprasit, P., Krisdhasima, V. and McGuire, J. The surface activity of  $\alpha$ -lactalbumin,  $\beta$ -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. The surface activity of  $\alpha$ -lactalbumin,  $\beta$ -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:** Interactions between milk proteins, starter cultures and hydrocolloidal milk fat replacers

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

Don McMahon, Dept. of Nutrition and Food Sciences,  
Utah State University

Mike LaFevre, Dept. of Nutrition and Food Sciences,  
Utah State University

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

**Objectives:**

In response to consumer demand, hydrocolloid fat replacers have been developed by many food companies. Some of fat mimetics may have properties that are useful to replace milk fat in cheese products. We hypothesize that fat replacers interact with milk proteins and dairy starter cultures that aid in developing fat-like characteristics to lowfat dairy products. To test this hypothesis, we will study interactions of milk protein, dairy starter cultures and milk fat replacers that are claimed to have milk fat mimetic properties. The specific objectives of this research are to:

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

**Results:**

Initial microbial characterization was done using three commercial starter cultures used for in production of 33% reduced fat Cheddar cheese. Studies to expand the number of useful starter cultures for lowfat cheese manufacture have been conducted by characterizing 45 other strains in the USU culture bank. Each strain metabolizes each fat replacer differently. Growth and acid production increase when starch-based fat replacers are added to milk and M17 media. However, protein-based fat replacers inhibit growth and slow acid production significantly.

A rapid semi-automated screening assay was developed to monitor glucosidase activity in starter cultures to aid in selecting useful cultures for use in lowfat cheese. Glucosidase activity is strain dependent and predicts the ability of starters to utilize

starch-based fat replacers. Additional culture characterization and identification was done using the Biolog system.

Lowfat cheese (50% reduced) has been made using different single starter strains in combination with each fat replacer. Each strain and fat replacer contributes different flavors to the final product. Control of acid production in the vat was difficult with starch-based fat replacers, as was predicted from the culture screening data.

**Impact of Research:**

Manufacture of lowfat cheese is difficult because limited starter strains are available that produce commercially acceptable cheese. Little information is available concerning use of fat replacers and their interaction with starter cultures. This research points out that strain selection for manufacture of lowfat cheese is critical for production of acceptable cheese.

**Publications:**

LeFevre, M., and B. Weimer. 1993. ADSA, College Park, Maryland.

**Project Title:** Production of extracellular proteases of *Brevibacterium linens* for use in lowfat cheese

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

Ben Dias, Dept. of Nutrition and Food Sciences,  
Utah State University

Soni Ummadj, Dept. of Nutrition and Food Sciences,  
Utah State University

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

**Objectives:**

*B. linens* has been established to produce a variety of serine proteases during different phases of growth depending on environmental conditions. We hypothesize that these organisms will be useful to produce lowfat cheese with acceptable flavor, body, and texture based on the action of these proteases. This hypothesis will be tested by conducting the following specific objectives:

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

**Results:**

Nine strains of *B. linens* have been screened for growth, aminopeptidase (AP), protease, lipase, and glucosidase activity with and without starch and protein-based fat replacers. All screened activities are strain and fat replacer dependent.

Maximum growth occurred between 36 and 48 h and remained in stationary phase for at least 96 h, when testing was terminated. Specific growth rates changed with milk fat and fat replacer concentration. Higher concentrations of fat replacers slowed the growth rate, while at lower concentrations fat replacers enhanced the growth rate of all strains.

Cellular location for different AP activities were determined with the exocellular and intracellular fractions demonstrating the highest activity. Intracellular activity varied with each strain tested. Strain 8377 contained the highest total activity while strain 9175 contained the least and was almost exclusively valine AP activity. Exocellular AP activity was high and all strains contained moderate arginine and lysine AP activity. No strains contained measurable proline or arginine carboxypeptidase activity. Total AP activity was comparable to that of *Lactobacillus casei* and *Lactobacillus helveticus* strains used in lowfat cheese manufacture commercially.

Two strains showed the highest extracellular protease activity which developed between 48 and 60 h of growth. The activity increased to 96 h when the testing was terminated. Studies are continuing to determine the effect of fat replacers on the activity of extracellular proteases.

Lipase activity was inversely related to the fatty acid chain length tested. This characteristic was strain dependent with 5/9 strains showing high total activity. Butyrate lipase activity was the highest in many stains. Stearate activity was the lowest or not detectable in all strains.

Glucosidase activity was strain dependent. Three strains contained  $\alpha$ - and  $\beta$ -galactosidase activity, suggesting that these strains can grow in milk via sugar metabolism. The other strains contained little or no glucosidase activity, indicating that they must use amino acids as the carbon source in milk if starch-based fat replacers are not added.

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

These data have provided information about the metabolic characteristics of *B. linens* that impact lowfat cheese flavor. Based on these data strain selection of *B. linens* for use in lowfat cheese production is critical to achieve an acceptable finished product. The strain selected for initial cheese trials produced an acceptable flavor at 1 mon, but at 2 mon the flavor was too bitter and rancid. Small scale cheese trials are continuing using different strain combinations to produce a balanced, aged 50% reduced Cheddar cheese.

#### **Publications:**

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

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#### **Publications:**



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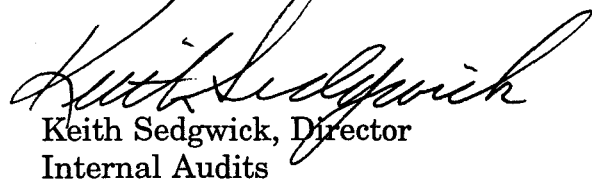
OFFICE OF INTERNAL AUDITS  
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October 25, 1993

To National Dairy Promotion  
and Research Board

QUALIFIED INTERNAL AUDITORS' STATEMENT

We have performed an accounting of the Annual Financial Report by Project,  
of the Western Dairy Research Center, from July 1, 1987 to June 30, 1993.

  
Keith Sedgwick, Director  
Internal Audits

Utah State University  
October 25, 1993

### Research Objective 3.1

**Investigate the contribution of the proteinase/peptidase enzyme systems of starter and non-starter bacteria to flavor, texture, and body development in low-fat cheeses.**

Future acceptance and increasing demand for low-fat cheese will be heavily dependent on the availability of starter cultures that produce a high-quality, flavorful product. Development of these cultures will in turn be dependent upon identification of key microbial enzymes and metabolites which promote desirable cheese flavor, texture, and body. To accomplish this requires characterization not only of proteinase enzymes, but also that of individual peptidases, lipases, and other key enzymes that contribute to cheese flavor, texture, and body.

### Research Strategy 3.1.1

**Selection of traditional lactococcal starter strains based on their hydrolysis of milk proteins for manufacture of low-fat cheese**

Low fat cheese manufacture presents unique demands for lactococcal starter cultures. Traditional strains, commercially available, have proven to produce inferior low-fat cheese even though they produce high quality full-fat cheese. One important difference between starters used in full-fat cheese and low-fat cheese is that strains used for low-fat cheese must produce acid at a slower rate. Derivation, widely used in New Zealand and Australia to produce phage resistant variants, produces many slow acid-producing mutants that have been discarded in the past. Phage derivation also changes many biochemical characteristics that influence proteolysis and subsequent flavor development. These changes also alter the required optimum growth parameters for starters in the production plant. To produce high quality low-fat cheese on an on-going commercial basis, 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.

- RKT
- USA  
WB
- USA  
RKT  
WB
- A. Select slow acid-producing starter strains (both *Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris*) that have proven quality records in Cheddar cheese manufacture. Selection will be compared to benchmark strains that are currently used for 33% reduced low-fat cheese.
  - B. Examine the effects of media and environmental parameters on growth, proteinase, and peptidase activity.
  - C. Characterize the effects of strain derivation for phage resistance on the phenotypic expression of lactose, proteinase, peptidase, and metabolism in parents and phage-resistant derivatives.

*Other*  
*of*  
*the*  
*proteins*  
*of*  
*the*  
*strains*

~~D~~ Examine the influences of heat-shock on proteinase and peptidase systems of these lactococcal starter strains.

Key Personnel:

Dr. Randall K. Thunell, Galloway  
West/CulTech, Adjunct Professor, USU  
Dr. Bart Weimer, Assistant Professor, USU



**Research Strategy 3.1.2**

**Improvement of low-fat cheese flavor and texture through identification, isolation, and analysis of proteolytic enzymes and metabolites produced by adjunct lactobacilli during accelerated Cheddar cheese development.**

- BW  
JB  
HSA JS  
UW  
HSA JS  
JB  
JB  
WJ  
WJ
- A. Characterize total peptidase and lipolytic enzyme activity in several *Lactobacillus* cheese adjunct cultures.
  - B. Isolate and characterize the gene for the general proteinase in *Lactobacillus helveticus* CNRZ 32.
  - C. Isolate and characterize genes for peptidase enzymes in the cheese adjunct *Lactobacillus casei* JB201.
  - D. Isolate and characterize the genes for diacetyl reductase and diacetyl synthase in the cheese flavor adjunct *Lactobacillus casei* JB202.
  - E. Characterize the influence of individual peptidases from *L. helveticus* CNRZ 32 on milk proteolysis and texture.
  - F. Track the microbial conversion of milk proteins into peptides, amino acids and flavor compounds in ripening cheese made with and without culture adjunct to identify key flavor and texture components which develop more rapidly in cheese made with *Lactobacillus* adjunct cultures.

Key Personnel: Dr. Jeff Broadbent - Assistant Professor, USU  
Dr. Bart Weimer - Assistant Professor, USU  
Dr. James Steele - Assistant Professor, CDR/UWM  
Dr. Mark Johnson - Senior Scientist, CDR

[The undertaking of this research objective represents a unique collaborative effort between the WCDPRT and the Center for Dairy Research at the University of Wisconsin-Madison.]

✓ 20  
**Research Strategy 3.1.3**

**Characterization, isolation, and analysis of stress proteins in dairy lactobacilli to improve ripening of low-fat cheese.**

Several studies have demonstrated the use of heat- or freeze-shocked *Lactobacillus* cheese starter adjuncts to accelerate cheese flavor development, but the molecular basis of this relationship remains unexplored. Lactobacilli are one of the few species of bacteria able to grow in ripening cheese. Growth studies in other bacteria suggest that survival under these conditions probably requires synthesis of stress proteins.

- A. Determine whether heat, low temperature, pH, or NaCl trigger a stress response in the commercial flavor adjunct cultures *Lactobacillus helveticus* R120, *Lactobacillus helveticus* R150, *Lactobacillus casei* subsp. *casei* LF302 and *Lactobacillus casei* subsp. *casei* LC20.
- B. Prepare heat-, freeze-, acid-, or NaCl-shocked populations of lactobacilli and use them as adjuncts in low-fat Cheddar cheese manufacture, then use taste panels, high pressure liquid chromatography, and capillary electrophoresis to characterize the resultant peptide profiles and compare these to flavor and textural properties of the cheese obtained from each of the treated populations of adjuncts.
- C. From data obtained in objectives 1 and 2, identify specific stress proteins that may be associated with improved low-fat cheese flavor and texture.
- D. Locate and isolate the genes that encode those proteins and study them at the molecular level.

Key Personnel: Dr. Jeff Broadbent, Assistant Professor, USU  
Dr. Craig Oberg, Professor, Weber State University, Ogden, UT

**Research Strategy 3.1.4**

**Isolate naturally-occurring lactococcal strains using genetic probes to improve the flavor and texture of low-fat Cheddar cheese.**

We have developed and applied genus-specific oligonucleotide probes to identify *Lactococcus lactis* subsp. *cremoris*. Subspecies-specific probes have also been developed that conserve differences in 16S ribosomal RNA between subspecies *lactis* and subspecies *cremoris* that make it possible to conclusively identify strains of these different subspecies. Because it is clear that differences between strains of lactococci are important in cheese texture and flavor it is now suggested that strains of *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* isolated from nature (plants and raw milk supplies from different countries) be evaluated for their use in making high quality low-fat Cheddar cheese.

- A. Characterize strains isolated from a variety of environments to ensure their identity to subspecies based on currently accepted biochemical tests, such as hydrolysis of arginine, growth at various pH, growth in various sodium chloride concentrations, and growth at different temperature levels.
- B. Continue to isolate strains from raw milk samples obtained from various parts of the world in order to develop a culture bank of *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* from as many different environments as possible.
- C. Analyze the peptide fractions produced by these various strains using capillary electrophoresis.
- D. Conduct pilot-scale Cheddar cheese manufacture (at either Tillamook County Creamery Association in Tillamook, Oregon or at the Utah State University's Gary H. Richardson Dairy Products Laboratory) using these isolated and characterized strains of *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*. The strains used will be those isolated as described above. We will conduct an expert evaluation of the flavor and texture of these cheeses.
- E. Develop laboratory parameters in addition to capillary electrophoresis which can be used to recognize strains of the isolated lactococci which would represent good candidates for use in low-fat Cheddar cheese manufacture.

Key Personnel: Dr. Stephen Giovannoni, Assistant Professor, OSU  
Dr. William E. Sandine, Professor, OSU  
Dr. Bart Weimer, Assistant Professor, USU  
Dr. Jeff Broadbent, Assistant Professor, USU  
Mr. Ed Yates, Production Mgr., Tillamook Cheese, OR  
Prof. Floyd Bodyfelt, Professor, OSU  
Mr. Randall Bagley, Dairy Plant Supervisor, USU

BW  
**Research Strategy 3.1.5**

**Production of extracellular proteinases of *Brevibacterium linens* for use in low-fat cheese.**

Proteinase enzyme systems from starter cultures are known to affect flavor and texture development in cheese. An alternative approach is to use proteinases from *Brevibacterium linens*, which are more active than those from lactic acid bacteria to accelerate body, texture, and flavor development in low-fat cheese.

- A. Develop a whey-based medium for the production of extracellular proteinases and peptidases by *Brevibacterium linens*.
- B. Determine the rate of proteolysis from proteinases produced by *Brevibacterium linens* in the presence coagulating enzymes in milk.
- C. Develop processing parameters required to produce Cheddar cheese with acceptable flavor and body using proteinases from *Brevibacterium linens*.

Key Personnel: B. C. Weimer, Assistant Professor, USU

### Research Objective 3.2:

**Investigate the contribution of addition of adjunct proteinases to protein breakdown and flavor development in low-fat cheeses.**

Adjunct proteinases can prove beneficial by hydrolyzing milk proteins specifically to develop desired flavor precursors and flavor compounds. Commercial proteinases (e.g. Neutrase) are available and have found use in some specific commercial dairy products. These proteinases and proteinase extracts will provide wide-ranging milk protein hydrolysis products.

### Research Strategy 3.2.1.

**Flavor and flavor development in low-fat cheddar cheese using adjunct proteinase and lipase extracts.**

The attainment of an appropriate or sufficient flavor profile in low-fat cheese prototypes has been a technical challenge for decades. Cheesemakers must either optimize or maximize the qualitative and quantitative dimensions of flavor in cheese made from low-fat milk. Interestingly, investigators in food science at Oregon State University in the early 1950s explored the proteolytic activity of extracts obtained from filberts (hazelnuts) on Cheddar cheese produced from whole milk. Ironically, the major problem encountered in their treatment of Cheddar cheese curd with extracted crude enzymes was the development of too much flavor intensity and bitter off tastes. Also, lipolytic activity was observed in the cheese trials with the crude filbert extracts. The enzyme addition level was not optimized and the process was not controlled, producing an objectionable flavor and aroma within a few days after.

By optimizing the selection, concentration, and addition of proteinase enzyme preparations, including the cheese manufacturing modifications that would be necessary for a controlled flavor development, we should be able to improve the development of cheese flavor in low-fat cheese. The final experiment corresponds to a preliminary trial for cheese making with these added enzymes in a commercial plant. This is considered necessary as the non-starter microflora from the plant will add important flavor notes to the aged cheese.

- A. Obtain a series of defined enzyme preparations with unique proteolytic and lipolytic activities.
- B. Determine the inactivation rate of the various enzyme extracts by temperature, pH, and salt concentration in a protein/fat cheese matrix.
- C. Treat Cheddar cheese curd with the enzyme preparations identified in (A) and evaluate the impact of enzyme supplementation on the structural, physical, and sensory properties of treated cheese relative to an untreated control.

- DF
- D. Evaluate technologies to arrest/control selected enzyme activities at specific times during cheese ripening. A primary consideration will be the use of ultra-high pressure treatment, ca. 1000-2000 atmospheres.
- E. Manufacture Cheddar cheese at 33, 45, 50, and 60% fat reduction with enzymes added to the milk, to the curd before cooking, or during milling and salting.
- CF
- F. Determine enzyme activities in cheese blocks (2" x 3" x 5") stored at 5 to 30°C for 120 days.
- EM
- G. Conduct sensory evaluation by a trained taste panel at days 7, 30, 60, 90 and 120.
- H. Interpret findings and perform final cheese-making experiment in a commercial plant (preferably).
- EN
- I. Conduct sensory evaluation by (1) a consumer taste panel and (2) a trained taste panel of the commercial plant cheeses after 60, 90 and 120 days of aging.

Key Personnel: Dr. J. Antonio Torres, Associate Professor, OSU  
Dr. Daniel F. Farkas, Professor, OSU  
Dr. Conly L. Hansen, Associate Professor, USU  
Professor Floyd W. Bodyfelt, Professor, OSU  
Dr. Michael H. Penner, Assistant Professor, OSU  
Scott Rankin, OSU

DA

### Research Objective 3.3

#### **Determine the role of proteins in the structure and physical properties of low-fat cheese.**

Consumption of cheese has grown steadily for the past ten years. Even with this type of growth there are still a number of areas in cheese sales that could be filled. One example is low-fat or nonfat Mozzarella cheese. Stretch and melt characteristics of these types 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, knowledge of why Mozzarella cheese stretches and melts is still very limited. An understanding of the process of Mozzarella cheese melting and stretching would enable development of new cheese products designed to satisfy consumer demands for low-fat and nonfat cheeses.

### Research Strategy 3.3.1

#### **Rheology and microstructure of low-fat Mozzarella cheese.**

- A. Objectives/Strategies supported by NDPRB Competitive Grant Program:
1. Study the microstructure changes that occur during the manufacture and storage of Mozzarella cheese using electron microscopy and correlate those changes with cheese melt and stretch properties.
  2. Develop thermophilic starter cultures, such as *S. salivarius ssp thermophilus*, *Lactobacillus helveticus* and *Lactobacillus casei*, that will be suitable for use in manufacture of low-fat Mozzarella cheese.
  3. Determine the effects of reduced fat and elevated moisture levels on the physical properties of Mozzarella cheese..
  4. Use the thermophilic starter cultures determined above as having the most appropriate properties to make low-fat Mozzarella cheese.
  5. Manufacture low-fat Mozzarella cheese using several stabilizers to bind water. The type and concentration of stabilizer will be optimized to produce the best stretch and melt over time.
  6. Replace some or all milk fat with commercial fat replacers and determine their ability to withstand elevated cooking and molding temperatures of Mozzarella cheese. Evaluate their effects on physical properties of the cheese, specifically stretch, melt, and cook color during refrigerated storage.





### **Research Objective 3.4.**

#### **Determine interactions between fat mimetics and milk components in low-fat cheese.**

Many food ingredient companies have responded to the demand for low-fat foods by developing fat mimetics. Simplese (The NutraSweet Co.), protein-based, and Stellar™ (Staley Manufacturing Co.), starch-based, are two types available. These non-fat substances should mimic milk fat globules with respect to size and be able to interact with milk proteins the way fat globules do. Different fat replacers have been developed for specific 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. We will examine how fat mimetics interact with milk components to replace milk fat globules and their functionality in improving flavor, body, and texture of low-fat cheese.

### **Research Strategy 3.4.1**

#### **Interactions between milk proteins and hydrocolloidal milk fat replacers**

- A. Determine size distributions of selected commercial fat replacers in a simulated milk buffer.
- B. Examine the rennet and acid coagulation characteristics of milk with fat removed and fat replacers added.
- C. Measure partitioning of fat replacers between curd and whey during milk coagulation and syneresis.
- D. Determine heat and pH stability of fat replacers in the presence of milk components.
- E. Determine characteristics of protein binding to surfaces of fat replacer particles.

Key Personnel: Dr. Bart Weimer, Assistant Professor, USU  
Dr. Don McMahon, Associate Professor, USU  
Dr. Mike LeFevre, Research Associate, USU

203  
**Research Strategy 3.4.2.**

**Investigate effects on traditional starter and non-starter cultures by fat mimetics.**

Background information about the use of commercial fat replacers in cheese making is not available. No reports have been published describing the functionality interactions between starter and non-starter cultures and milk fat replacers. Addition of fat replacers to milk creates a new dimension to the microbiology of fermented dairy products. Interactions between fat replacers, starter, and non-starter cultures are unknown. Fat replacers offer to starter and non-starter bacteria new sources of growth nutrients that are not normally found in a cheese matrix. Growth and acid production by starter cultures will be influenced differently by protein- and starch-based fat replacers during cheese production. Functionality of fat replacers will be affected by the proteinases, peptidases and metabolic end-products of lactic acid bacteria.

- A. Determine the interaction of the rate of proteolysis from proteinases produced by starter and non-starter cultures in the presence of fat mimetics and coagulating enzymes in milk.
- B. Develop and describe processing parameters required to produce Cheddar cheese with acceptable flavor and body using milk fat mimetics and proteinases from starter and non-starter cultures.

**Key Personnel:** Dr. Bart Weimer, Assistant Professor, USU

### Research Objectives 3.5

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

The difference in the rate of starter die-off and growth of non-starter bacteria affect the production of key enzymes involved in flavor development of cheeses. We have shown that microbial activities are affected by diffusion phenomena in the cheese matrix and this is a possible mechanism for modifying the contribution of starter and non-starter bacteria to flavor development in low-fat cheese.

Kinetic analysis will be conducted with respect to temperature during the cooling of freshly formed cheese blocks and during aging. Mathematical modeling will be used to select experimental conditions to reduce the number of temperature conditions studied. Both fast and slow cheese cooling mass are undesirable.

#### Research Strategy 3.5.1

#### **Effect of Temperature and Fat on Microbial Kinetics of Starter and ~~Non-Starter~~ Bacteria in the Fat-Protein Matrix of Low-fat Cheese.**

Adjunct.

- ABC
- A. Manufacture cheese at 0, 33, 45, 50, and 60% fat reduction and determine starter die-off and non-starter growth in cheese blocks (2" x 3" x 5") during storage at 5 to 30°C.
- B. Conduct a kinetic analysis of data obtained in (A).
- C. Determine possible mechanisms for the observed behavior.
- CD
- D. Apply the kinetic models to the cooling and aging of 40 lb. and 640 lb. cheese blocks to estimate microbial population changes as affected by fat content (FDW), moisture content (MNFS), block size, and cooling/aging temperature treatments.
- EF
- E. Apply the kinetic models for starter die-off and non-starter growth to the cooling and aging of 40 lb. and 640 lb. cheese blocks to estimate microbial population changes as affected by fat content (FDW), moisture content (MNFS), block size and cooling/aging temperature treatments.
- F. Develop a simplified kinetics analysis to be used as a screening device to aid in the selection of low-fat cheese cultures.
- GW
- G. The culture combinations selected as suitable for use in low-fat cheeses under Research Objective 3.1 will be evaluated using the procedures described above.

Key Personnel: Dr. J. Antonio Torres, Associate Professor, OSU  
Dr. Conly L. Hansen, Associate Professor, OSU  
Dr. Paul A. Savello, Assistant Professor, USU  
Dr. Bart Weimer, Assistant Professor, USU  
Mr. Randall Bagley, Dairy Plant Supervisor, USU

DF  
**Research Strategy 3.5.2**

**Texture and Flavor Development in High-Pressure Treated Low-fat Cheddar Cheese.**

High pressure inactivates microbial cells at rates dependent on the bacteria type and environmental factors (such as pH, temperature,  $a_w$ ). Such pressure inactivation is not cheese-block location dependent as the pressure is the same everywhere in the block. This would allow for better control of non-starter bacteria than relying just on temperature reduction. Furthermore, 1 to 3 decimal reductions in nonstarter bacteria populations, with minor or no reduction in pressure-resistant starter strains, would give time to reduce the temperature of the Cheddar cheese block to a value at which the growth of non-starter bacteria could occur at a more desirable rate. In addition, the pressure treatment may release key enzymes involved in flavor development, an important consideration in low-fat Cheddar cheese.

Preliminary experiments at OSU on protein matrixes have shown that textural properties change with pressure treatment and so, the effect of pressure (50, 500, 1,100, 3,000 and 5,000 atm) on texture development during aging will be measured by objective methods. A formal trained panel evaluation does not seem feasible at this time, because size of the high pressure chamber limits the number of samples that can be prepared. An expert panel will conduct preliminary objective textural evaluations before using trained panel evaluators.

- A. Determine inactivation rate of starter and non-starter bacteria in a cheese matrix as affected by temperature, pressure, and time.
- B. Select optimum conditions for pressure-treating cheese.
- C. Make cheese (full fat and 50% reduced fat) and determine the effect of pressure treatment on starter and non-starter bacteria over 120 days of storage at 5, 12, 15, and 20°C.
- D. Measure changes in sensory properties and texture of the pressure treated and control cheeses during storage of the above cheeses.
- E. Interpret the findings from (C) and (D) as to the suitability of using high pressure treatment in the manufacture of low-fat cheeses and conduct scale-up if applicable.

Key Personnel:      Dr. J. Antonio Torres, Associate Professor, OSU  
                             Dr. Daniel F. Farkas, Professor, OSU  
                             Dr. Conly L. Hansen, Associate Professor, USU  
                             Professor Floyd Bodyfelt, Professor, OSU

### Research Strategy 3.5.3

#### **Fat Reduction Effect on the Water Interaction with the Protein-Fat Matrix.**

Several studies have demonstrated large moisture gradients in Cheddar cheese blocks, particularly in 640 lb. blocks. These moisture gradients represent large physicochemical environment differences in the protein-fat matrix and affect microbial and chemical kinetics of aroma, flavor, and texture development during aging. Industrial measurements indicate even more important moisture differences in reduced fat Cheddar cheese. A recently completed OSU-USU study showed that moisture gradients are driven by temperature differences within a block, however the exact mechanisms remain to be determined and may involve: (1) flow of unbound water through the protein-fat matrix; (2) chemical potential differences associated with temperature gradients; and (3) chemical potential differences associated with chemical composition differences caused by the temperature/moisture difference. An understanding of the exact contribution of these mechanisms and as they are affected by fat reduction level is essential to control/minimize moisture gradients within the protein-fat matrix.

- A. Prepare Cheddar cheese at 33, 50, and 60% fat reduction.
- B. Determine at constant temperature, water activity and water bound to the protein matrix and by competition to lower molecular weight solutes (NaCl, sodium lactate, amino acids, small peptides, and organic acids) during storage at 5, 12, 15, 20, 25, and 30 °C.
- C. Define water activity and moisture content in cheese slabs stored under conditions of a temperature gradient through the slab as affected by temperature treatment and location in the cheese.
- D. Develop a moisture transfer model that takes into account the moisture transfer mechanisms identified above and then determine appropriate model parameters.
- E. Validate the moisture transfer model by determining moisture content in 40 and 640 lb. blocks of Cheddar cheese with 0, 33, 50, and 60% fat reduction level as affected by location in the block and aging time.
- F. Make process recommendations for the manufacture of low-fat cheese..

Key Personnel:     Dr. J. Antonio Torres  
                          Dr. Conly L. Hansen



**WESTERN DAIRY CENTER  
for Dairy Protein Research  
and Technology**

**ANNUAL MEETING**  
Utah State University, Logan  
July 30, 1993

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WESTERN CENTER FOR DAIRY PROTEIN  
RESEARCH AND TECHNOLOGY

MINUTES OF  
ANNUAL MEETING 1992

July 9-10, 1992  
Corvallis, Oregon

The 1992 Annual Meeting of the Western Center for Dairy Protein Research and Technology (WCDPRT) was held on July 9 and 10, 1992 on the campus of Oregon State University (Corvallis, Oregon). Following research progress reports by Principal Investigators to members of the Operational Advisory Committee (OAC), the business meeting was held on July 10, 1992.

OAC Members in Attendance: Robert Bursey, Janet Collins-Williams, Barbara Blackistone, Paul Savello, Rodney Brown, Floyd Bodyfelt, Donald McMahon, Boyd Gardner, Gale Moser, Sheldon Pratt, Raj Narasimmon, James Moran, Douglas Willrett.

Many visitors from the WCDPRT research institutions were in attendance.

The meeting opened with introductory comments by the Director, Paul Savello. The Director stated that the WCDPRT had a successful year 1991-92 in terms of its research efforts. The minutes of the 1991 OAC meeting of the Western Center for Dairy Foods Research were accepted by voice vote.

Barbara Blackistone described the funding breakdown of the NDPRB among the various divisions of the NDPRB (including nutrition/product research, marketing, advertising, export committees). Barbara described the projected increased funding to the research centers. Each center, including the WCDPRT, will received \$500,000 total funding starting FY94. Barbara detailed that this funding consists of two parts: \$400,000 base funding to the center with the additional \$100,000 funding being granted when the center indicates (through its Annual Report) that the objectives/strategies of the Annual Plan were met and appropriately conducted.

The organizational objectives of the NDPRB were explained by Barbara. These objectives of the NDPRB were formulated by the different divisions of the NDPRB with the express intent to meet the present and future needs and the dairy industry. The organizational objectives are: milkfat utilization, fluid milk products, dairy innovation, and enhancing perceptions of a quality diet. Clarification of these organizational objectives relative to dairy center objectives/focuses was made by Barbara Blackistone and Bob Bursey. The four NDPRB organizational objectives have equal weight and the WCDPRT should concentrate on which organizational objectives into which it can best fit, rather than trying to fit into all four objectives.

The financial report of the WCDPRT was presented by the Director. A large balance was indicated because a low number of proposed projects was approved during the year. Another reason was that a large number of projects terminated because FY92 was the end of the original five-year contract period.

The Director informed the OAC that all Principal Investigators of the WCDPRT have been required to first send research proposals to the Competitive Research Program Office of the NDPRB before submitting the same proposals to the Center. Of twenty one (21) proposals sent the NDPRB in January 1992 for competitive program review, five (5) research proposal ideas were awarded to WCDPRT researchers. (Two of the five research projects were combined into one larger project, making four total awards.)

The Director presented his response to the California Milk Advisory Board (CMAB) letter that followed a CMAB meeting in Boise, Idaho. The CMAB letter discussed a consolidation of the "research centers of the West." The Director responded by letter to Mr. Ralph Watts (of CMAB) with copies sent to the Utah Dairy Commission, United Dairymen of Idaho, and the Oregon Dairy Products Commission.

The Director presented his view that the WCDPRT has become a "mini-competitive" research center. Researchers at the WCDPRT have been competing among themselves for the funds available within the WCDPRT. The Director stated that this may not be the best way to conduct the research at the center because the basic concept of a research center is to effectively use the expertises and facilities of all involved in center research. The Director mentioned that the NDPRB and USDA already have competitive research programs to which any and all researchers are welcomed to approach for such competitive funding. The Director stated that the WCDPRT should consider a small re-structuring so that the competitive nature of internal WCDPRT funding is minimized.

The Director presented his idea of WCDPRT "Program Research" and funding. This "program research" concept will be based on team approach to research programs at USU/BYU and at OSU for the period 1993-96. The Director stated that the research program goals are both basic and applied with publishing the basic findings and protecting intellectual property of applied aspects. The Director also described the research programs should be based on "quantifiable objectives on a yearly basis." The annual meeting/reports should update WCDPRT supporters of research progress, protection, technology transfer/translation.

The Director presented an "incentive" concept to promote more contributions to the center's programs. This concept was to provide OSU with funds taken from the WCDPRT to match (in some multiple) the Oregon Dairy Products Commission contribution.

The Director also presented a "Mini-Competitive Research" concept that would provide funding to principal investigators within the center, based upon review for scientific merit and WCDPRT objectives. This concept would fund projects that would fit the needs of the WCDPRT supporters and to research expressed needs of the dairy industry within the center's region.

Responses to the Director's initiatives included:

- "good idea to identify real problems and have researchers go after them, but mechanism to do so is pudgy"
- "this is very similar to the original concept of the dairy center"
- "the WCDPRT could also be a source of matching funds for other funding agencies' initiatives"
- "team concept of research would be a big change for an academic institution"
- "it appears that the director wishes more discretion to speed up the funding process of proposed research"

- “there is always the possibility that the proposed structure of the center will not work”
- “could it be possible to have proposal dates always be open as long as the proposal comes from a team of researchers”
- “industry likes the teamwork approach in which the goal is identified then the research is told to get there whereas academia does not function that way”
- “teamwork functions better as a product is brought closer to commercialization”
- “teamwork probably does not function best when basic science problems are being investigated”
- “the California center’s use of short-term and long-term research has had better acceptance by the industry”

Motion by McMahon: The Director should take comments of the OAC to formulate and implement a program to streamline funding of center research and to encourage cooperative research between/among researchers of the center.

Motion seconded.

Douglas Willrett added that the teamwork approach to center research should be explored. The NDPRB competitive grant program still provides a vehicle for researchers to compete for research funds in another research area.

Motion passed by unanimous vote.

The Director presented the new WCDPRT research proposals to the OAC, informing the committee of suggested budget changes in and TAC comments of the proposals where appropriate.

The Director explained how United Dairymen of Idaho (UDI) funds (to the WCDPRT) will be used to fund Dr. Schelling’s project at the University of Idaho. The Director stated that during a March 1992 meeting of the UDI in Boise, Idaho the use of such UDI funds would be used in a “directed research” effort to the University of Idaho.

A discussion followed regarding the possibility of State of Washington’s and Washington State University’s (WSU) involvement in the WCDPRT. The Director explained to the OAC that he had a telephone conversation with Dr. Don Lee of WSU about a year earlier about this possibility. Dr. Lee had asked for a guarantee that State of Washington promotion association money remain in that state and that NDPRB money could be obtained in the form of a match so that dairy research could be conducted at WSU. The Director informed Dr. Lee that the WCDPRT did not function under such a protocol and that all funds contributed to the center is placed in a “pool” with research funds awarded to principal investigators/institutions based on technical merit and meeting the objectives of the center and the NDPRB. Floyd Bodyfelt explained to the OAC that the possible support money from the State of Washington is slightly different because the main dairy association in the state performs significant amount of in-house research.

Motion by Boyd Gardner: To approve the recommended new projects for the WCDPRT.

Motion seconded.

Motion passed by unanimous vote.

The date was set for the 1993 Annual Meeting of the WCDPRT. The meeting will be held on July 29 and 30, 1993 at Logan, Utah.

The Director stated that an honorarium was paid to all TAC members (excluding the Director) for their work in reading, reviewing, and evaluating the twenty-one research proposals submitted to them by the WCDPRT. The honorarium is a small payment for the effort spent by the TAC to help ensure that meritorious research is conducted at the center.

Motion by Rod Brown to adjourn the 1992 Annual Meeting of the WCDPRT.

Motion seconded.

Motion passed by unanimous vote.

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**WESTERN CENTER FOR DAIRY PROTEIN  
RESEARCH AND TECHNOLOGY  
1993 ANNUAL MEETING AGENDA  
UTAH STATE UNIVERSITY**

FRIDAY, July 30, 1993, Nutrition and Food Sciences, Room 202

7:30 a.m. Registration; continental breakfast

8:00 a.m. Welcome and introductions  
Paul Savello, Director

**PRACTICAL RESEARCH**

8:15 a.m. Identifying batch of origin of finished cheese made in continuous processes.  
PI: Lynn Ogden (BYU)

8:30 a.m. Comparative effects of whey protein concentrate (WPC), lactose, salt, phosphate, and pH on cooked yield, bind, and acceptability of turkey rolls and boneless hams.  
PI: Daren Cornforth (USU)

8:45 a.m. Evaluation of iron-protein complexes in iron-fortified dairy products.  
PI: Arthur Mahoney (deceased)(USU)  
Presenter: Mohan Reddy

9:00 a.m. Estimation of individual milk proteins and genetic variants by multicomponent analysis of amino acid profiles.  
PI: Rodney Brown (USU)

9:15 a.m. Using a natural 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.  
PI: G. R. Schelling (UI)

9:30 a.m. Using whey for improvement of exposed subsoils and sodic and saline-sodic soils.  
PI: Conly Hansen (USU)

9:45 a.m. Effects of iron fortification on chemical, physical, microbiological and nutritional properties of yogurt.  
PI: Donald McMahon (USU)  
Presenter: Sharareh Hekmat (USU)

**10:00 a.m. BREAK**

**PRACTICAL ( continued)**

10:15 a.m. Extrusion processing of milk proteins.  
PI: Conly Hansen (USU)

10:30 a.m. Rheology and microstructure of Mozzarella cheese.  
PI: Donald McMahon (USU)

- 10:45 a.m. Development of high protein low-fat fermented foods from yogurt cheese.  
PI: Conly Hansen (USU)  
Presenter: Yehia A. El-Samragy (USU)
- 11:00 a.m. Development of a process for production of UF milk retentate powder.  
PI: Conly Hansen (USU)  
Presenter: Yehia A. El-Samragy (USU)
- 11:15 a.m. Controlling age gelation of UHT sterilized milk concentrates.  
PI: Donald McMahon (USU)  
Presenter: Mohamed A. Mohamed (USU)
- 11:30 a.m. Function of whey proteins and lactose in age gelation of UHT-processed milk concentrate - Part 2.  
PI: Donald McMahon (USU)  
Presenter: M. Christopher Alleyne (USU)

**NOON LUNCH**

**FUNDAMENTAL RESEARCH**

- 1:00 p.m. Cloning the nisin and other genes of lactic streptococci into *Leuconostoc* species and amplification of nisin production.  
PIs: Jeffery Broadbent (USU) and William Sandine (OSU)
- 1:30 p.m. Growth of Bifidobacteria in milk: Association with *Streptococcus thermophilus* and *Lactobacillus* species as measured by genetic and enzymatic probes.  
PI: William Sandine (OSU)  
Presenter: Joseph W. Booth (OSU)
- 1:45 p.m. Characterization of milk proteolysis by lactococcal starter culture strains using amino acid analysis.  
PI: Rodney Brown (USU)
- 2:00 p.m. Bacteriophage-resistance gene replacement in *Lactococcus lactis*.  
PI: B Geller (OSU)
- 2:15 p.m. Influence of preadsorbed protein on adhesion of *Listeria monocytogenes* to dairy food contact surfaces.  
PI: Mark Daeschel (OSU)

**LOW-FAT CHEESE RESEARCH**

- 2:30 p.m. Interactions between milk proteins, starter cultures and hydrocolloidal milk fat replacers.  
PI: Bart Weimer (USU)
- 2:45 p.m. Production of extracellular proteases of *Brevibacterium linens* for use in lowfat cheese.  
PI: Bart Weimer (USU)
- 3:00 p.m. **BREAK**

**3:15 p.m. Operational Advisory Committee Business Meeting**

**6:00 p.m. Steak fry, NFS lawn.**

**7:00 p.m. FESTIVAL OF THE AMERICAN WEST.**



# WESTERN DAIRY FOODS RESEARCH CENTER

## 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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**WESTERN DAIRY FOODS RESEARCH CENTER**

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# WESTERN DAIRY FOODS RESEARCH CENTER

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**Project Title:** Identifying Batch of Origin of Finished Cheese Made in Continuous Processes

**Personnel:** Lynn V. Ogden, Dept. of Food Science and Nutrition.  
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Salvador U. Parco, Dept FSN, BYU

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

**Objectives:**

Use of cheddaring and blockforming towers in cheese manufacturing has considerably increased the efficiency of cheese plants. A serious quality control problem created by this semi-continuous process is difficulty in tracking batches. Current equipment and procedures for tracking batch of origin require that all towers be full at the end of the transfer of each batch to the towers and that all towers run at the same rate. Many plants can not use the system because of the impracticality of those conditions. When one batch is substandard and batch tracking is poor, the equivalent of three batches are downgraded to assure that all of the substandard cheese is included when the Commodity Credit Corporation buys cheese. When selling to the private sector the problem is similar. Either excessive numbers of blocks have to be recalled and checked to identify the substandard cheese or excessive numbers of blocks are sold as distressed cheese to avoid expensive checking. The potential is high that some low quality cheese will make it on the market and damage the products reputation.

The objective of this project is to develop a system to track batches through pressing towers. We were to evaluate the suitability of the Hunter Qual Probe as a sensor and turmeric or additional annatto as a marker in alternate batches of cheddar cheese. If feasible, it is a further objective to develop a system applying the concept. If not feasible, it is a further objective to search for and assess other sensor technologies for this application.

**Results:**

Less than acceptable reliability using the colorimetric approach has reported in 1992 and attention was turned to assessment of other sensor approaches. The reliability of two commercially available Ultrasonic instruments in partially evacuated environment were evaluated. One of the instruments read distances reliably to 5.8 meters at 381 mm Hg vacuum. The Ultrasonic instruments did not perform as well in the actual tower. Although the vacuum was less than 254 mm Hg, distances readings became unreliable at greater distances than 10 feet. We concluded that ultrasonic sensors would not be reliable enough for this application. Concern about the lack of compatibility with the harsh conditions inside the tower also raised doubts as to the durability of such a system.

A thermal dissipation approach was conceived and explored in which all sensors would be outside the tower. Mounted outside the inner skin of the tower, resistance heaters and thermistors sense how quickly applied heat is dissipated from the stainless steel skin. More rapid dissipation

indicates that cheese is on the inside and is conducting heat away. A buildup of heat indicates that no cheese (heat dissipating mass) is on the other side of the skin. A string of prototype sensors were fabricated and mounted on the outside of a stainless steel milk can. They were able to differentiate the presence of absence of water in the can and a light display indicated the level of water in the can. It is expected that a computerized system that scans a string of sensors and measures the rate of temperature rise using very short time increments and very small currents, could be a very reliable sensor and could be linked to a computerized control system that could not only track batches but also control the depth of cheese in the tower.

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

Inability to accurately identify the batch of origin of specific units of cheese for recall or downgrading is a problem in plants using cheddaring and/or block forming towers in cheese manufacturing. One plant manager estimated the unnecessary value reduction on his cheese to be \$250,000 per year assuming that 6.5 million pounds of cheese was unnecessarily downgraded and an average reduction in value of \$.04 per pound. Ability to identify batch of origin would prevent that loss.

#### **Publications:**

Parco, S.U. and L.V. Ogden. 1992. Batch determination in semi-continuous cheesemaking operations using on-line colorimetry. *J. Dairy Sci.* 75(supp 1): 127.

**Project Title:** Comparative Effects of Whey Protein Concentrate and Phosphate on Bind and Acceptability of Turkey Rolls

**Personnel:** Daren Cornforth, Department of Nutrition & Food Sciences, Utah State University

Brent Dobson, Department of Nutrition & Food Sciences, Utah State University

Igor Moiseev, Department of Nutrition & Food Sciences, Utah State University

**Funding:** Western Center For Dairy Protein Research And Technology

**Objectives:**

Phosphates (primarily sodium tripolyphosphate) are widely used to increase the bind strength and cooked yield of processed meat products. However, phosphates are relatively expensive, and are slow to dissolve in brines. There is also evidence that phosphates in foods reduce the absorption of zinc, calcium, and iron (Mahoney and Hendricks, 1978; Zemel and Bidari, 1983). Phosphates are not permitted as ingredients in cooked poultry products imported into Japan. Thus, there is some interest in the development of alternative binding agents for cooked meat products. Milk proteins have potential as alternative binding agents in cooked meats. We have previously shown that both nonfat dry milk and whey protein concentrate will increase the bind strength of turkey rolls, compared to controls made without milk proteins (Dobson et al., 1993). Thus, the objective of this study was to compare bind strength of turkey rolls made with phosphate or with various commercially available whey protein concentrates.

**Results:**

Turkey rolls (90% breast meat, 10% thigh meat, 10% added water, 1% salt ) were made with no WPC or phosphate (control), sodium tripolyphosphate (0.5%), or WPC (1 or 3%). All percentages were based on meat weight. Three types of WPC were compared;

- WPC-50 (50 % protein)
- WPC-60 (60% protein, acidified to pH 4.5)
- WPC-75 (75% protein, pH 7.0, "high-gel")

Rolls made with phosphate had significantly higher bind strength and firmer texture than controls or rolls made with WPC. Rolls made with 1%WPC-75 also had higher bind strength than controls. Rolls made with 1% WPC-60 had the lowest bind strength and cohesiveness. Rolls with 1% WPC-50 or 1% WPC-75 had moderate turkey flavor. In general, rolls made with 3% WPC had lower scores for intensity of turkey flavor. We have previously shown that nonfat dry milk inhibits the pink discoloration that sometimes develops during refrigerated storage of turkey rolls

(Dobson and Cornforth, 1992). In this study however, no color differences were observed among treatments and controls.

**Conclusions:**

Use of high-gel WPC produced rolls with higher bind and acceptability than controls, but not as high as rolls made with phosphate. WPC-50 and acidified WPC (WPC-60) were unacceptable as binding agents in turkey rolls.

**Publications:**

Dobson, B. N. and Cornforth, D. P. 1992. Nonfat dry milk inhibits pink discoloration in turkey rolls. *Poultry Sci.* 71:1943.

Dobson, B. N., Moiseev, I. V., Cornforth, D. P., Savello, P., Wood, R. J. and Andersen, R. 1993. An instrument for measuring bind strength of restructured and emulsion-type meat products. *J. Texture Studies.* In press.

Mahoney, A. W. and Hendricks, D. G. 1978. Some effects of different phosphate compounds on iron and calcium absorption. *J. Food Sci.* 43:1473.

Zemel, M. B. and Bidari, M. T. 1983. Zinc, iron, and copper availability as affected by ortho phosphates, polyphosphates and calcium. *J. Food Sci.* 48:567.

## FINAL REPORT

**Project Title:** Evaluation of Iron-Protein Complexes in Iron-Fortified Dairy Products

**Personnel:** Dr. Arthur W. Mahoney (deceased)

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  
Utah Agriculture Experiment Station

**Project Period:** July 1, 1989 to December 31, 1992

### Objectives:

The primary objective of this study is to determine the chemistry of iron-protein complexes in milk as related to cheese making. The mechanism and thermodynamics of iron-protein complex formation is necessary to understand the functional and stability characteristics of iron-milk protein complexes. Elucidation of the mechanism would provide the information to enable the dairy processor to use conditions and/or competitive reagents to maximize complex formation. The specific objectives of the project are to:

1. To determine the nature of interaction of individual milk proteins, viz.,  $\alpha_{S1}$ -casein,  $\beta$ -casein,  $\kappa$ -casein,  $\beta$ -lactoglobulin,  $\alpha$ -lactalbumin, and bovine serum albumin with ferric iron at pH 6.60 and possible side chain residues involved in the complex formation.
2. To study the effect of pH, NaCl concentration, Ca(II), and dephosphorylation of protein on the binding of iron(III) to  $\alpha_{S1}$ -casein (major casein). Effect of pH on the possible side chain residues involved in the complex formation.
3. To determine the effect of iron binding on the conformation and self-aggregation of different milk proteins at pH 6.60 and at different pHs for  $\alpha_{S1}$ -casein.
4. To study the effect of ferric chloride on the chymosin hydrolysis and rennet clotting time of milk .
5. To study the effect of ferric chloride addition to milk on the physico-chemical properties of casein micelles, such as, iron(III), calcium and phosphorous content, voluminosity, and surface hydrophobicity.
6. To determine the catalytic potency of iron-milk protein complexes on oxidative damage to model lipids.



## Methodology:

1. Binding of iron(III) to milk proteins was measured by diafiltration method using Fe(III)-Nitrilotriacetic Acid(NTA).
2. Possible iron(III)-binding groups in proteins were determined by monitoring visible-difference spectra of iron(III)-protein complexes in the visible region (330 to 700 nm).
3. Iron(III)-induced conformational changes in the proteins were monitored by ultraviolet-difference spectroscopy and fluorescence spectroscopy, and iron(III)-induced aggregation of proteins was monitored by FPLC on Superose-6 gel filtration column (30 x 1.0 cm).
4. Chymosin hydrolysis in milk was followed by monitoring the release of macropeptide and rennet clotting time by Formagraph.
5. Calcium and iron were determined by Atomic Absorption Spectroscopy.

## Results:

**Objective 1: Binding of Iron(III) to  $\alpha_{S1}$ -Casein,  $\beta$ -Casein,  $\kappa$ -Casein,  $\beta$ -Lactoglobulin,  $\alpha$ -Lactalbumin, and Bovine Serum Albumin at pH 6.60.** Binding of iron(III) to individual protein fractions was studied by diafiltration method and binding isotherms are shown in Figure 1. Binding data analyzed by Scatchard equation are given in Table I. Binding of iron(III) to all the proteins studied increased as the free iron(III) concentration increased. Scatchard analysis indicated that both casein and whey protein fractions (except  $\beta$ -LG and  $\alpha$ -LA) have two groups of non-identical binding sites with differing affinities for binding iron(III). It appears that first set of binding sites ( $n_1$ ) are preferentially filled, compared to the second set of binding sites ( $n_2$ ). The values of free energy change ( $\Delta G$ ) calculated using the relationship,  $\Delta G = -RT \ln K$ , for different iron(III) binding sites in casein and whey protein fractions were negative and low in magnitude (Table I), indicating that the complex formation between iron(III) and protein fractions is spontaneous and thermodynamically favorable. Relative binding of iron(III) to different casein and whey protein fractions follows the order:  $\alpha_{S1}$ -CN >  $\beta$ -CN > BSA >  $\kappa$ -CN >  $\beta$ -LG >  $\alpha$ -LA.

**Visible-Difference Spectra of Iron(III) - Protein Complexes at pH 6.60.** Difference absorption spectra of iron(III)-protein complexes in PIPES (10mM)-NaCl (100mM) buffer, pH 6.60 were carried out in order to determine the possible amino acid side chain groups involved in the binding of iron(III) to different proteins. Difference absorption spectra of iron(III)- $\alpha_{S1}$ -CN and iron(III)- $\beta$ -CN complexes have negative absorption bands in 420-421nm region and positive absorption bands in 470-471nm region, 491-492 nm region, and in 560-562 nm region ; iron(III)- $\kappa$ -CN complexes have positive absorption bands in 423-425 region, 470-471nm region, 491-492 nm region and 560-562 nm region; and iron(III)-BSA, iron(III)- $\beta$ -LG and iron(III)- $\alpha$ -LA complexes have positive absorption bands in 421-422 nm region, 470-471 nm region, 491-492 nm region, and 568-570 nm region. The model spectra of the complexes of phosphorylserine, Asp, Glu and other amino acids with iron(III)

revealed that the negative absorption band in 420 nm region was due to phosphorylserine-iron(III) complexes and the positive band in 565 nm was due to and carboxyl-iron(III) complexes, where as the positive bands in 420, 470 and 490 nm region were possibly due to a chelate site involving carboxyl, nitrogen and oxygen groups. This may indicate that phosphoserine groups are the major iron(III) binding sites in  $\alpha_{S1}$ -CN and  $\beta$ -CN, where as carboxyl groups are the major iron(III) binding sites in  $\kappa$ -CN, BSA,  $\beta$ -LG, and  $\alpha$ -LA.

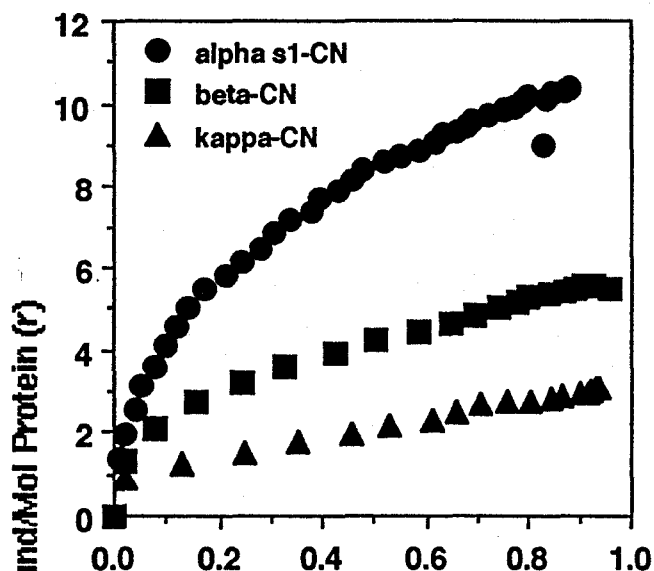


Fig. 1A. Binding Curves of Fe(III) to Caseins, pH 6.60,  $\mu=0.1$

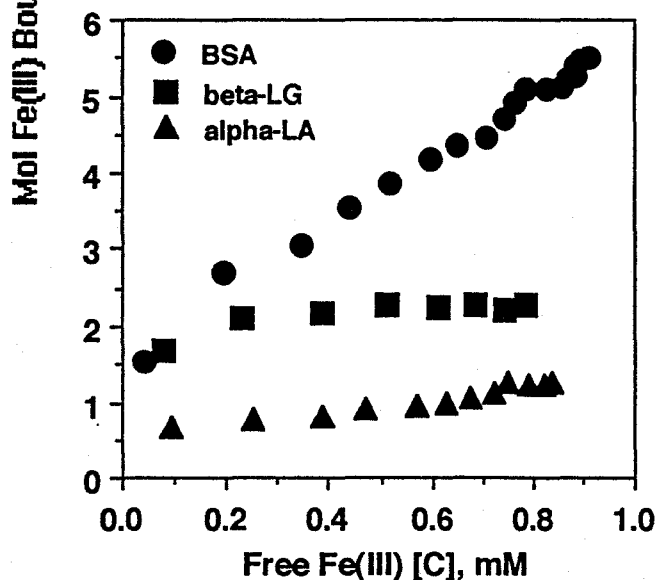


Fig. 1B. Binding Curves of Fe(III) to Whey Proteins

**Objective 2: Effect of pH, NaCl, Ca(II) and Dephosphorylation of Protein on the Binding of Fe(III) to  $\alpha_{S1}$ -Casein.** Binding of iron(III) to  $\alpha_{S1}$ -casein was studied as a function of pH (5.6, 6.1, 6.6, 7.2, and 7.8), NaCl concentration (0.1 and 0.5 M), and dephosphorylation of protein using diafiltration method and the binding data analyzed by Scatchard equation. pH and NaCl had no influence on the number of iron binding sites on the protein, which remained constant at  $n=20$  (Table II). However, binding affinity of iron(III) to protein decreased with an increase in pH from 5.6 to 7.8 and NaCl from 0.1 to 0.5 M (Table 2). Thus, from the practical point of view, the binding affinity of iron(III) increases as

the pH of milk is lowered by microbial action during cheese making. Dephosphorylation of  $\alpha_{S1}$ -casein decreased the number of iron binding sites on the protein (Table 2) indicating that phosphoserine groups play a major role in the binding. In another experiment, partial displacement of protein-bound Ca(II) by iron(III) was observed for  $\alpha_{S1}$ -casein. This indicates that iron(III) successfully competes for some of the Ca(II) binding sites on the protein. Free energy change ( $\Delta G = -RT \ln K$ ) calculated for the binding of iron(III) to  $\alpha_{S1}$ -casein under different pHs and NaCl concentrations was negative and low in magnitude (-3.79 and -7.28 k cal M<sup>-1</sup>) indicating that the binding of iron(III) to protein is instantaneous and thermodynamically favorable.

Table I. Thermodynamic Parameters for the Binding of Iron(III) to Milk Proteins (pH 6.6,  $\mu=0.1$ , 24°C)

Protein	Fe to protein molar ratio	Number of binding sites ( <i>n</i> )	Binding constants ( <i>K</i> ) (M <sup>-1</sup> x 10 <sup>3</sup> )	Free energy change ( $\Delta G$ ) (k. cal. M <sup>-1</sup> )
$\alpha_{S1}$ -CN	4.7	14.0 (2.7, 4.5, 6.8)	128 to 2.9	-6.9 to -4.7
$\beta$ -CN	4.8	9.0 (2.7, 2.0, 4.3)	44 to 1.3	-6.3 to -4.2
$\kappa$ -CN	3.8	6.0 (1.5, 4.5)	88 to 1.0	-6.7 to -4.1
BSA	13.3	8.5 (3.5, 5.0)	20 to 1.8	-5.8 to -4.4
$\beta$ -LG	3.7	2.4	31	-6.1
$\alpha$ -LA	2.8	0.9	32	-6.1

**Effect of pH and Dephosphorylation on Visible-Difference Spectra of Iron(III) -  $\alpha_{S1}$ -Casein Complexes.** Difference absorption spectra of iron(III)-protein complexes in the visible region (370 to 750 nm) were carried out to determine the possible amino acid side chain groups involved in the binding of iron(III) to  $\alpha_{S1}$ -casein at different pHs (5.6 to 7.8). Negative absorption band in 420 nm region, and positive absorption bands in 470, 490 and 560 nm region were observed for the protein at all pHs. The model spectra of the complexes of iron(III) with phosphorylserine, Asp, Glu and other amino acids revealed that the negative absorption band in 420 nm region was due to iron(III)-phosphorylserine complexes and the positive band in 565 nm region was due to iron(III)-carboxyl complexes, where as the positive bands in 420, 470 and 490 nm region were possibly due to a chelate site involving carboxyl, nitrogen and oxygen groups. When  $\alpha_{S1}$ -casein was dephosphorylated the negative absorption band in 420 nm region disappeared and a positive absorption band appeared in its place, further confirming that the negative absorption band in 420 nm region is contributed by iron(III)-phosphorylserine complexes. The magnitude of the negative absorption band in 420 nm region decreased, where as the positive absorption band in 470, 490, and 565 nm region increased with an increase in pH. This may indicate that the involvement of carboxyl groups in the complex formation increased as the pH is moved away from their isoelectric pH. Thus, phosphorylserines and carboxyl groups of Asp and Glu seem to play a major role in the binding of iron(III) by  $\alpha_{S1}$ -casein.

Table II. Thermodynamic Parameters for the Binding of Iron(III) to  $\alpha_{S1}$ -Casein at 24°C

Fe to Protein Molar Ratio	pH	NaCl (mM)	Number of Binding Sites ( <i>n</i> )	<i>K</i> (M <sup>-1</sup> ) x 10 <sup>3</sup>	$\Delta G$ (k. cal. M <sup>-1</sup> )
9.4	5.6	100	20 (8.0, 7.0, 5.0)	22 to 2.5	-5.9 to -4.6
9.4	6.1	100	20 (7.7, 12.3)	8 to 1.7	-5.3 to -4.4
9.4	6.6	100	20 (5.2, 4.8, 10)	46 to 1.0	-6.3 to -4.1
9.4	7.2	100	20 (4.8, 15.2)	24 to 1.1	-5.9 to -4.1
9.4	7.8	100	20 (4.6, 15.4)	21 to 0.6	-5.9 to -3.8
4.7	6.6	100	14 (2.7, 4.5, 6.8)	128 to 2.9	-6.9 to -4.7
4.7	6.6	500	14 (2.2, 4.5, 7.3)	227 to 2.0	-7.3 to -4.5
9.4	7.8*	100	9 (4.0, 5.0)	35 to 1.2	-6.2 to -4.2

\*Dephosphorylated  $\alpha_{S1}$ -casein

**Objective 3: Iron(III)-Induced Conformational Changes in  $\alpha_{S1}$ -Casein,  $\beta$ -Casein,  $\kappa$ -Casein,  $\beta$ -Lactoglobulin,  $\alpha$ -Lactalbumin, and Bovine Serum Albumin at pH 6.60.**

Conformational changes in proteins, especially, changes in the environment of aromatic side chains in the proteins as a result of binding of iron(III) were monitored by following fluorescence emission after excitation at 280 nm. Addition of iron(III) caused a decrease in fluorescence emission intensity together with a red shift of the emission maximum for the proteins, except  $\beta$ -lactoglobulin. Iron(III) addition to  $\beta$ -lactoglobulin caused a decrease in emission intensity without affecting the emission maximum. The results indicate that binding of iron(III) to proteins perturbed the environment of aromatic amino acid (Trp and Tyr) residues possibly by exposing them to a more polar environment.

Since, binding of metal ions (Ca, Mg, Ba, Zn) to caseins has been reported to induce aggregation of proteins, it was of interest to see if iron(III) induces similar changes in milk proteins. Therefore, Iron(III)-induced aggregation of  $\alpha_{S1}$ -casein,  $\beta$ -casein,  $\kappa$ -casein,  $\beta$ -lactoglobulin,  $\alpha$ -lactalbumin, and bovine serum albumin at pH 6.60 was followed by FPLC on Superose-6 gel filtration column (30 x 1.0 cm). The results indicated that the binding of iron(III) to milk proteins did not induce aggregation. This may be due to the differences in the nature of binding of iron(III) (which forms co-ordinate complexes) to proteins compared to other bivalent metal ions (which form ionic complexes).

**Effect of pH on Iron(III)-Induced Conformational Changes in  $\alpha_{S1}$ -Casein.** Intrinsic fluorescence studies at pH 6.60 indicated that iron(III) binding to  $\alpha_{S1}$ -casein perturbed the environment of aromatic amino acid residues in the protein. Therefore, the iron(III)-induced conformational changes in the protein were further probed at different pHs by following UV-difference spectra and fluorescence emission spectra. The UV-difference spectra of  $\alpha_{S1}$ -casein induced by iron(III) at pH 5.6, 6.1, 6.6, 7.2, and 7.8 had absorption bands at 310 nm indicating the possible involvement of tryptophan residues in charge-transfer type

complex formation with iron(III). Since it is characteristic of charge-transfer type complexes to quench tryptophan fluorescence in proteins, the accessibility of tryptophanyl residues in  $\alpha_{S1}$ -casein in the absence and presence of iron(III) was followed by iodide quenching at pH 6.6. Intrinsic tryptophan fluorescence of  $\alpha_{S1}$ -casein excited at 295 nm was quenched by iodide indicating the partial exposure of tryptophan residues in protein. Addition of iron(III) to protein caused further quenching of tryptophanyl fluorescence by iodide. This further supports the UV spectral data that binding of iron(III) to protein brought about changes in the immediate vicinity of tryptophan environment. This could be due to the involvement of tryptophan residues in charge-transfer type complex formation with iron(III), thus exposing them to a more polar environment. Addition of Ca(II) to  $\alpha_{S1}$ -casein induces progressive aggregation as shown by a net increase in fluorescence emission intensity and a blue shift in fluorescence maximum. However, iron(III) does not seem to induce any aggregation, instead seems to form soluble iron(III)-protein complexes.

Fluorescence emission of  $\alpha_{S1}$ -casein (excited at 280 nm) at different pHs (5.6 to 7.8) was followed as a function of iron(III) concentration (0 to 0.2 mM). Addition of increasing concentrations of iron(III) brings about a red shift of the emission maximum, together with a decrease in fluorescence intensity at all pHs. Fluorescence intensity of  $\alpha_{S1}$ -casein as a function of iron(III) concentration was fitted to a modified Stern-Volmer plot and quenching constant ( $K_Q$ ) was calculated from the slope. Quenching constant was high at pH 5.6 ( $1.1 \times 10^4 \text{ M}^{-1}$ ) and decreased with an increase in pH ( $0.65 \times 10^4 \text{ M}^{-1}$  at pH 7.8). This indicates that iron(III) binding had maximum effect on the conformation of protein at pH 5.6 than at pH 7.8. Since conformational changes in general affect functional properties of proteins, it was of interest to see if binding of iron(III) affects Ca(II) sensitivity of  $\alpha_{S1}$ -casein; addition of iron(III) to protein increased its Ca(II) sensitivity.

**Objective 4: Effect of Ferric Chloride on Chymosin Hydrolysis and Rennet Clotting Time of Milk.** Since binding of iron(III) to caseins induced conformational changes, it was of practical interest to see if addition of ferric chloride to milk during cheese making would affect renneting properties. Therefore, ferric chloride (0 to 0.86 mM, about 0.2 mM concentration is normally employed making iron-fortified Cheddar cheese) was added to cold milk before pasteurization and to pasteurized at 30°C (as in the case of regular cheese making process) and chymosin hydrolysis and rennet clotting time (RCT) were evaluated. Iron(III) at all the concentrations employed had no effect on the chymosin hydrolysis of either milks. However, iron(III) decreased the RCT of both whole and skim milk when added to cold milk before pasteurization and increased when it was added to pasteurized skim milk, but it barely affected the RCT of pasteurized whole milk.

**Objective 5: Effect of Ferric Chloride Addition to Milk on the Iron, Calcium and Inorganic Phosphorous Contents, Voluminosity, and Surface Hydrophobicity of Casein Micelles.** Ferric chloride at 0, 0.43 and 0.86 mM concentration was added to cold milk before pasteurization and to pasteurized milk under constant stirring and allowed to equilibrate for 30 min. The milk was then centrifuged at 100,000 x g to partition the casein micelles from whey and the iron, calcium and phosphorous contents and voluminosity of micelles was determined. Surface hydrophobicity of casein micelles was determined by adding 0.2 mM ANS to milk before centrifugation and by estimating the distribution of ANS

in micellar and whey phases by measuring ANS fluorescence. Addition of ferric chloride to either milks resulted in a decrease in voluminosity and calcium content, and an increase in iron and inorganic phosphorous content of micelles with an increase in ferric chloride concentration in milk. However, addition of ferric chloride to milk before pasteurization increased the surface hydrophobicity of micelles and addition of ferric chloride to milk after pasteurization decreased the surface hydrophobicity of micelles. This may explain why RCT of milk decreased when ferric chloride was added to milk before pasteurization, and it increased when ferric chloride was added to milk after pasteurization.

**Objective 6: Catalytic Potency of Iron-Milk Protein Complexes on Oxidative Damage to Model Lipids.** Off-flavor production due to iron-induced lipid peroxidation is a major concern in fortifying dairy foods with iron. Hence, it is of practical interest to know the catalytic potency of iron(III)-casein and iron(III)-whey complexes on iron-catalyzed lipid peroxidation. Therefore the inhibitory effect of casein and whey protein fractions on iron-catalyzed lipid peroxidation was studied in soy lecithin-iron(III)-ascorbate model system by estimating TBARS (nmol MDA per mg lipid). The results indicated that both casein and whey protein fractions inhibited iron-induced lipid peroxidation in proportion to their iron(III)-binding capacity.

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

Iron fortification would increase the iron intakes of people who consume large amounts of dairy products, and it would allow people concerned with their nutriture to consume larger amounts of dairy products to achieve greater calcium intakes. Thus, dairy products would be even more healthful in the diet if iron-fortified. This research will provide basic information on the mechanisms of iron binding to proteins in dairy products, information essential to industrializing the technology of fortifying dairy products with iron.

#### **Publications:**

Reddy, I. M. and Mahoney, A. W. Effect of  $\text{FeCl}_3$  on chymosin hydrolysis and rennet clotting time of milk. *J. Dairy Sci.* 75, 2648, 1992.

Reddy, I. M. and Mahoney, A. W. The effect of iron on the renneting of milk. *J. Dairy Sci.* 74 (Suppl. 1), 100, D-57, 1991.

Reddy, I. M. and Mahoney, A. W. Binding of Fe(III) to bovine  $\alpha_{S1}$ -casein. *J. Dairy Sci.* 74 (Suppl. 1), 100, D-58, 1991.

Reddy, I. M. and Mahoney, A. W. A study of the interaction of Fe(III) to bovine  $\alpha_{S1}$ -casein using ultraviolet and fluorescence spectroscopy. *J. Dairy Sci.* 74 (Suppl. 1), 100, D-59, 1991.

Reddy, I. M. and Mahoney, A. W. Characterization of Fe(III) - milk protein complexes. *In* Brief Communications and Abstracts of Posters of the XXIII International Dairy Congress, Vol. I, 253, P-471, 1990.

## **Publications (Contd.)**

Reddy, I. M. and Mahoney, A. W. (1993). Diafiltration and visible spectroscopic study of the binding of iron(III) to bovine  $\alpha_{S1}$ -,  $\beta$ -, and  $\kappa$ -caseins, bovine serum albumin,  $\beta$ -lactoglobulin, and  $\alpha$ -lactalbumin at pH 6.60. (In Review)

Reddy, I. M. and Mahoney, A. W. (1993). A visible spectroscopic study of iron(III)-amino acid complexes at pH 6.60. (In Review)

Reddy, I. M. and Mahoney, A. W. (1993). Iron(III)-induced conformational changes in bovine  $\alpha_{S1}$ -,  $\beta$ -, and  $\kappa$ -caseins, bovine serum albumin,  $\beta$ -lactoglobulin, and  $\alpha$ -lactalbumin at pH 6.6 studied by ultraviolet and fluorescence spectroscopy. (In Preparation)

Reddy, I. M. and Mahoney, A. W. (1993). Effect of pH, NaCl, CaCl<sub>2</sub>, and dephosphorylation of protein on the binding of iron(III) to  $\alpha_{S1}$ -casein. (In Preparation)

Reddy, I. M. and Mahoney, A. W. (1993). A study of the interaction of iron(III) to bovine  $\alpha_{S1}$ -casein using ultraviolet and fluorescence spectroscopy. (In Preparation)

Reddy, I. M. and Mahoney, A. W. (1993). Inhibition of iron-catalyzed lipid peroxidation by casein and whey protein fractions. (In Preparation)

## **Papers Presented at Conferences:**

Reddy, I. M. and Mahoney, A. W. Effect of Iron on the renneting of milk. Presented at 86th ADSA Annual Meeting, Utah State University, Logan, August 12-15, 1991.

Reddy, I. M. and Mahoney, A. W. Binding of Fe(III) to bovine  $\alpha_{S1}$ -casein. Presented at 86th ADSA Annual Meeting, Utah State University, Logan, August 12-15, 1991.

Reddy, I. M. and Mahoney, A. W. A study of the interaction of Fe(III) with bovine  $\alpha_{S1}$ -casein using ultraviolet and fluorescence spectroscopy. Presented at 86th ADSA Annual Meeting, Utah State University, Logan, August 12-15, 1991.

Reddy, I. M. and Mahoney, A. W. Characterization of Fe(III) - milk protein complexes. Presented at 'The 23rd International Dairy Congress', Montreal, Canada, October 7-12, 1990.

## **Short Courses:**

Dr. Mohan Reddy attended a ACS lecture-laboratory short course on 'Microemulsions, Vesicles, and Liquid Crystals' at Clarkson University, Potsdam, NY during July 19-24, 1992 to develop methodology at Utah State University.

**Theses/Dissertations:** Nil.

**Patents:** Nil.

**Project Title:** Estimation of Individual Milk Proteins and Genetic Variants by Multicomponent Analysis of Amino Acid Profiles

**Personnel:** Rodney J. Brown, Dept. of Nutrition and Food Sciences, Utah State University.

Carol M. Hollar, Dept. of Nutrition and Food Sciences, Utah State University.

**Funding:** Western Dairy Foods Research Center

**Objectives:**

The overall objective is to determine proportions of specific milk proteins, groups of proteins and selected genetic variants of milk and other dairy products using information obtained from amino acid analysis, fast protein liquid chromatography and isoelectric focusing of samples. Completing the following specific objectives will enable the overall objective to be met.

1. Determine concentrations of groups of proteins in milk such as caseins or whey protein and the concentrations of specific milk proteins:  $\alpha_{S1}$ -,  $\alpha_{S2}$ -,  $\beta$ -, and  $\kappa$ -caseins,  $\alpha$ -lactalbumin,  $\beta$ -lactoglobulin, bovine serum albumin using amino acid analysis.
2. Separate genetic variants of specific milk proteins, and use amino acid analysis to quantify individual variants in a protein mixture.
3. Use techniques developed in 1-2 to analyze milk and other dairy products.
4. Determine mathematical procedures to obtain the most accurate and reproducible methods for estimating milk protein concentrations.

**Results:**

Percentages of  $\kappa$ -casein,  $\beta$ -casein,  $\alpha_{S1}$ -casein, and  $\alpha_{S2}$ -casein obtained with isoelectric focusing, cation-exchange fast protein liquid chromatography, and anion-exchange fast protein liquid chromatography compare well with published results. Isoelectric focusing and anion-exchange fast protein liquid chromatography methods separated  $\kappa$ -casein into its A and B variants. Isoelectric focusing and cation-exchange fast protein liquid chromatography separated  $\beta$ -casein into its A<sup>1</sup>, A<sup>2</sup>, and B variants.



Although amino acid analysis can identify whether  $\beta$ -casein A<sup>1</sup>, A<sup>2</sup>, or B variants are present, it cannot identify whether  $\kappa$ -casein A and B variants are present. Stepwise regression equations derived to predict samples as homozygous or heterozygous for  $\kappa$ -casein A and B and  $\beta$ -casein A<sup>1</sup>, A<sup>2</sup>, and B had coefficient of determination values of .18, .58, .82, and .72 for  $\kappa$ -casein A and B,  $\beta$ -casein A<sup>1</sup>,  $\beta$ -casein A<sup>2</sup>, and  $\beta$ -casein B.

### **Impact of Research:**

The goal of this project is to determine proportions of specific milk proteins (down to the level of specific genetic variants) or groups of proteins in milk and other dairy products from the information contained in a single amino acid analysis of a sample. As profit margins get tighter, the more closely milk supplied by farmers meets the requirements of dairy manufacturers becomes more important. Manufacturers prefer receiving milk that results in improved cheese yield and processing characteristics. Developing techniques to separate and quantify caseins and their genetic variants will help provide a more desirable milk supply to manufacturers.

### **Publications:**

Hollar, C. H., R.J. Brown and J. F. Medrano. 1990. Quantification of casein genetic variants by RP-HPLC, PAGE, and amino acid analysis. 85<sup>th</sup> American Dairy Science Association Meeting *J. Dairy Sci.* 73:Supp. 1, 80.

Hollar, C. M., A. J. R. Law, D. G. Dalgleish, and R. J. Brown, 1991, Separation of major casein fractions using cation-exchange fast protein liquid chromatography. *J. Dairy Sci.* 74:2403-2409.

Hollar, C. M., A. J. R. Law, D. G. Dalgleish, and R. J. Brown, 1991, Separation of major casein fractions using cation-exchange fast protein liquid chromatography. 86<sup>th</sup> American Dairy Science Association Meeting *J. Dairy Sci.* 74:Supp. 1, 91.

Hollar, C. M., A. J. R. Law, D. G. Dalgleish, J. F. Medrano, and R. J. Brown, 1991, Separation and quantification of  $\beta$ -casein A<sup>1</sup>, A<sup>2</sup> and B using cation-exchange fast protein liquid chromatography. *J. Dairy Sci.* 74:3308-3313.

Hollar, C. M., A. J. R. Law, D. G. Dalgleish, J. F. Medrano, and R. J. Brown, 1991, Separation of  $\beta$ -casein A<sup>1</sup>, A<sup>2</sup> and B using cation-exchange fast protein liquid chromatography. 86<sup>th</sup> American Dairy Science Association Meeting *J. Dairy Sci.* 74:Supp. 1, 103.

Hollar, C.M. 1992. Estimation of Selected Milk Protein Genetic Variants by Multi-Component Analysis of Amino Acid Profiles. Ph.D. Dissertation, Utah State University, Logan.

Hollar, C. M., and R. J. Brown, 1992, Separation and quantification of  $\alpha_{S1}$ -casein,  $\beta$ -casein A<sup>1</sup>, A<sup>2</sup> and B and  $\kappa$ -casein A and B using isoelectric focusing, and cation-exchange and anion-exchange fast protein liquid chromatography. 87<sup>th</sup> American Dairy Science Association Meeting *J. Dairy Sci.* 75:Supp. 1, 121.

Hollar, C. M., and R. J. Brown, 1993, Quantification of  $\alpha_{S1}$ -casein,  $\alpha_{S2}$ -casein,  $\beta$ -casein A<sup>1</sup>-5P, A<sup>2</sup>-5P, and B-5P, and  $\kappa$ -casein A-1P and B-1P using isoelectric focusing and cation- and anion-exchange fast protein liquid chromatography. Submitted for Publication.

Hollar, C. M., and R. J. Brown, 1993, Neuraminidase treatment of whole casein to improve the separation of  $\kappa$ -casein A-1P and B-1P using anion-exchange fast protein liquid chromatography. Submitted for Publication.

Hollar, C. M., and R. J. Brown, 1992, Estimation of  $\kappa$ -casein A-1P and B-1P and  $\beta$ -casein A<sup>1</sup>-5P, A<sup>2</sup>-5P, and B-5P using multi-component analysis of amino acid profiles. Internal Review Completed.

**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

**Objectives:**

The oxidation of milk causing off-flavors continues to be a problem. 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, but current nutritional management of dairy cows relative to vitamins A and E have a definite impact upon the tocopherol content of milk. The specific objectives of this phase of the study are as follows.

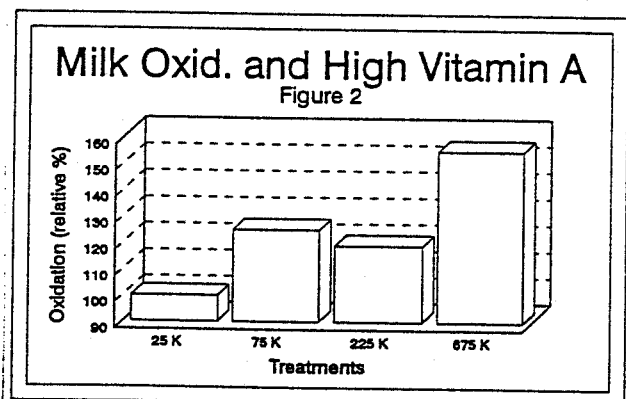
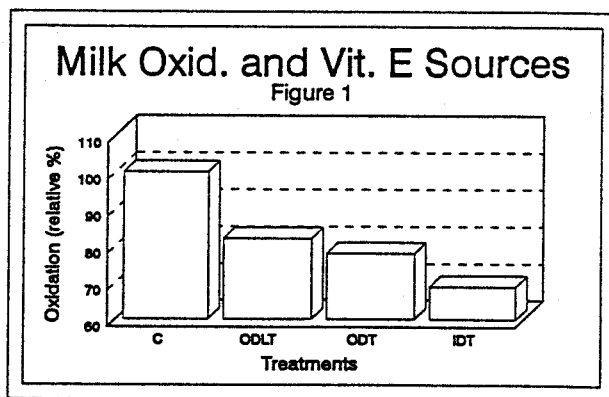
1. To evaluate the effect of various chemical and/or administration forms of vitamin E on milk vitamin E content and milk oxidation.
2. To determine the efficiency of gastrointestinal tract absorption and mammary transfer of vitamin E to milk.
3. To determine the effect of higher dietary levels of vitamin 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 late lactation 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. The milk sampling schedule involved 14 samples collected from day -2 through 22, with the treatments being given on day 0. Both the oral and injectable sources elevated milk alpha-tocopherol by day 1 and peaked on day 2. The oral doses produced elevated levels for 5 day, and the injectable for 9 day. The peak alpha-tocopherol concentrations in the milk at day 2 for treatments C, ODLT, ODT and IDT were .64, .72, .98 and 1.73 mg/l ( $P < .0001$ ). The total alpha-tocopherol secretion in the milk due to the treatments was determined by calculating a baseline for each cow based on average values for day -2, -1, 0, 14 and 22. 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 data indicate a relatively low transfer (3.01 %) of injected d-alpha-tocopherol across the mammary gland into milk. Furthermore, the transfer of vitamin E sources from the digestive tract to milk (.36 and .75 % for dl-alpha-tocopheryl acetate and micellized d-alpha-tocopherol respectively) would indicate a digestive tract absorption of 12.9 and 26.8 % for these two vitamin E sources assuming the same post-absorptive utilization efficiency as for the injectable vitamin E source. The rate of oxidation was measured over an appropriate time period on milk samples with added ferric sulfate by using the thiobarbituric acid (TBA) procedure. Figure 1 shows the decreased oxidation of milk as milk vitamin E is increased via more available vitamin E forms. Relative milk oxidation was significantly ( $P < .01$ ) reduced from 100 % for the control (C) to 82.1, 77.8 and 68.6 % for the oral dl-alpha-tocopheryl acetate (ODLT), oral micellized d-alpha-tocopherol (ODT) and injectable d-alpha-tocopherol (IDT) treatments respectively.



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 124.6, 118.8 and 154.9 % for the medium, high and very high vitamin A treatments respectively.

### **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.* (Abstract).

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*. Adelaide, Australia.

**Project Title:** Use of Cheese Whey for Soil Improvement

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

Darwin Sorensen, Professor and Mark Roginske, Graduate Student, Civil and Environmental Engineering Dept., Utah State University

**Funding:** Western Dairy Foods Research Center

**Objectives:**

Main

This project is a continuation of a project started in 1991. In this aspect of the project we will determine if the optimal application rates for reclamation of sodic and saline-sodic soils is harmful to the environment.

Specific

- Determine the extent crops and soil remove nutrients and COD load from land applied whey.
- Determine the best management practices for utilizing the soluble carbon in the whey as an energy source to reduce nitrate to ammonia and/or  $N_2$  gas as a means of keeping nitrate from entering subsurface waters.
- Determine maximum safe whey land application rate.
- Determine if odor will be a problem in land application of whey and/or how to avoid an odor problem.
- Determine value of whey for land treatment, i.e. from nutrients, pH adjustment, increasing aggregate stability and exposed subsoil reclamation.
- Determine the effects of total salts and salt ratios in whey, on soil physical conditions and on crop growth.

**Results:**

Soil gas samples were collected on 2/16, 2/26, 3/27, 4/17, 6/29, 7/9, and 7/10 for soil oxygen, carbon dioxide, and nitrogen percentage determination by gas chromatography (GC). As of 4/17,  $CO_2$  levels have been between 0.07 and 4.70% with most levels being under 1.00%. Oxygen levels have been between 15.70% and 21.80% and nitrogen levels range from 76.3 to 86.2%. Soil gas samples on 6/29, 7/8, and 7/10 have been run on the GC, but percentages have not yet been computed.

Soil sampling will begin in the fall.

Test Plot Observations Growth on alfalfa plots 1, 2, and 11 receiving the most amount of whey appeared stunted shortly after the 3/29 application. At the time of the first hay cutting (6/15), alfalfa on plots 1, 2, 4, and 12, along with control plot 9, appeared stunted. Remaining plots appeared taller and greener than the rest of the field. At the time of the second hay cutting (7/20), all plots that received whey on 7/8 showed non-uniform growth with some dead alfalfa spots, especially around the instrument areas. Control plots looked similar to the rest of the field.

Whey Application Whey from Cache Valley Cheese was applied to alfalfa test plots as follows:

<u>Date</u>	<u>Plot #</u>	<u>Amount/plot (gal)</u>
1-14-93	1,2,4,6,7,8,10,11,12	196
2-18-93	1,2,4,8,11,12	196
3-29-93	1,2,11	196
7-8-93	1,2,4,6,7,8,10,11,12	196

Alfalfa test plot numbers 3, 5, and 9 are control plots and will not receive whey.

Instrumentation Instruments in the ground at the time of the January, February, and March whey applications were ceramic cups, two at 2 feet and two at 3.5 feet, and soil gas points, one at 2 feet and one at 3.5 feet, for each plot. Four redox probes were installed in each plot at a depth of 30 cm by the time of the July whey application. Oxygen probes will not be installed in any of the plots and the oxygen probe order has been cancelled. Thermistors have been received and will be installed as soon as possible.

Sample Collection and Testing Soil solution samples were collected on 2/13, 2/24, 3/25, 4/16, 6/29, and 7/10 for COD, total Kjeldahl-N,  $\text{NH}_4\text{-N}$ ,  $\text{NO}_3\text{-N}$ , total phosphorus,  $\text{PO}_4\text{-P}$ , iron, manganese,  $\text{SO}_4$ , and  $\text{Cl}^-$  testing. Standard curve generation for  $\text{NO}_3\text{-N}$ ,  $\text{PO}_4\text{-P}$ ,  $\text{SO}_4$ , and  $\text{Cl}^-$  testing by ion chromatograph (IC) has been unsuccessful because of a leaking pump on the IC. A part has been ordered and sample testing will begin as soon as a proper standard curve can be made. Acceptable low level COD standard curves have been generated in replicate and sample testing will begin this week. Generation of an acceptable ferrous iron standard curve by the ferrozine colorimetry method has not been successful. Problems have been narrowed to dirty glassware and are being corrected. Manganese, total iron, total phosphorus, total Kjeldahl-N, and  $\text{NH}_4\text{-N}$  testing will be initiated as time allows.

Impact of Research:

Whey, particularly acid whey, has been considered a by-product of cheese manufacture with value that fluctuates widely with market conditions. Even at the present time, a high percentage of whey is thrown away. The goal of this project is to develop a new market for whey. Use of whey as a soil amendment and fertilizer coincides with the Low Input Sustainable Agriculture (LISA) goals.

Whey can be utilized to reclaim unproductive soils. Technology exists to reclaim sodic and saline sodic soils with expensive chemicals costing up to \$1,000.00/acre. Therefore, land owners can often buy new land for less than it will cost to reclaim these soils. The salt content and pH of acid whey, combined with its fertilizer value makes this an ideal material to reclaim these soils and provides a new market for a dairy product. The value of whey will be enhanced by creating this new demand and at the same time decrease its disposal by undesirable methods.

One of the most attractive aspects of this project is the cooperation between the Idaho dairy industry, Utah State University and the USDA-ARS Soil and Water Management Research Unit, Idaho. This project is receiving high visibility in Idaho and will serve as an example of what the WCDPRT does for the dairy farmer.

The information gleaned from this project will also make it possible to legally apply whey to land. Currently, many states including Idaho and Utah limit to a great extent land application of whey because of lack of application guidelines. Whey must either be dried or treated using some other, often expensive method so it can be sold or considered as wastewater and disposed of at considerable expense because of its high biochemical oxygen demand and solids content.

#### **Publications:**

Jones, S.B., C. W. Robbins, C.L. Hansen. 1993 Sodic Soil Reclamation Using Cottage Cheese (Acid) Whey. *Arid Soil Research and Rehabilitation* 7:51-61.

Jones, S.B., C.L. Hansen, C. W. Robbins. 1993 Fate of Chemical Oxygen Demand from Cottage Cheese (Acid) Whey Applied to a Sodic Soil. *Arid Soil Research and Rehabilitation* 7:71-78.



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

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

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

**Objectives :**

(brief statement on the overall goal of the project)

1. To produce low-fat and non-fat iron-fortified yogurt using FeCl<sub>3</sub>, Fe-casein or Fe-whey protein complex as the iron sources.
2. To determine growth and viability of Lactobacillus delbruekii ssp. bulgaricus and Streptococcus salivarius ssp. thermophilus in iron-fortified yogurt.
3. To determine iron binding sites using Elemental Filtering Electron Microscopy.
4. To determine the best procedure for making a high-quality iron-fortified yogurt.

**Results :**

**Objective 1.** Low-fat (2%) and non-fat iron-fortified yogurt with three sources FeCl<sub>3</sub>, Fe-casein and Fe-whey protein at three levels (10, 20, and 40 mg/Kg) for each sources were successfully made. The two iron sources, Fe-casein and Fe-whey protein were prepared by adding .5 M FeCl<sub>3</sub> into skim milk and cottage cheese whey respectively. The Ferrozine assay was used to determine percent iron recovery of the iron sources. The percent iron recovery of Fe-casein and Fe-whey protein were 46.76 and 88.6 respectively.

**Objective 2.** The yogurt cultures were obtained from Heart to Heart corporation. Enumeration of L. delbruekii ssp. bulgaricus and S. salivarius ssp. thermophilus were done using MRS (pH 5.4) and M17 media respectively. Lactic acid counts for non-fat iron fortified yogurt (FeCl<sub>3</sub>, Fe-casein and Fe-whey protein at 10, 20, 40 mg/Kg) started with 10<sup>8</sup> and in some cases 10<sup>9</sup> CFU/ml for each bacteria. Their number decreased only slightly after one month of storage. There were no significant

differences in bacterial counts between iron-fortified yogurt and the control. The bacterial counts for low fat iron-fortified yogurt is still in progress.

**Objective 3.** Several samples of iron-fortified yogurt were prepared for Scanning Electron Microscopy (SEM). Results indicated that preparation procedure for SEM did not wash away iron and localization of the bound iron was possible. However, Transmission Electron Microscopy (TEM) is necessary for more detail study of bound iron. The initial sample preparation for TEM which involved fixation with glutaraldehyde and osmium tetroxide and embedding with Standard Mollinbauer Araldite mixture (EPON) did not maintain iron in a binding state and localization of iron was not possible. Therefore, further investigations are required for obtaining the best procedure to keep the bound iron in the sample. This may involve utilizing different fixative, dehydrating reagent, transition reagent, embedding media, etc. Our goal is to focus on obtaining an optimum condition which would not cause removal of iron from the sample.

#### **Objective 4.**

**I. Iron Analysis :** The Ferrozine assay was used to quantitate iron in the iron-fortified yogurt after complete wet-ashing with nitric acid and Hydrogen Peroxide. The results from non-fat iron-fortified yogurt indicate uniform distribution of iron throughout the yogurt matrix for all of the sources ( FeCl<sub>3</sub>, Fe-casein and Fe-whey protein). The Analysis of low-fat iron fortified yogurt is still under investigation.

**II. Thiobarbituric Acid Test :** Initially distillation method was used to quantitate lipid oxidation materials in iron fortified yogurt. However, due to reaction of thiobarbituric acid with yogurt components (phospholipids, protein residues, sugar, etc.), an intense yellow color was present in all distillates. According to some investigators, the indicator 'pink' color could be extracted with cyclohexanol, but in case of yogurt, the yellow color was also extracted into cyclohexanol layer. Therefore, a new spectrophotometric method was used to determine lipid oxidation in the yogurt. The results indicates no significant differences between iron-fortified yogurt and control samples for non-fat iron fortified yogurt over one month of storage. The analysis of low -fat yogurt is still in progress.

**III. Sensory Analysis :** Quality of iron-fortified yogurt will be evaluated for appearance, texture, oxidizing off flavors (including oxidized, chemical and metallic flavors) by trained and untrained panelists during the second year of the project.

### **Impact of Research :**

We have shown that yogurt can be fortified with iron without affecting starter culture growth. Sensory evaluations are to be conducted during this coming year. If iron fortification does not adversely affect the flavor profile of yogurt, it would be possible to make iron fortified yogurts suitable for commercial manufacture.

### **Publications :**

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

Hekmat, S. and D. J. McMahon. 1993. Effects of iron fortification on chemical, physical and microbiological properties of yogurt. *J. Dairy Science* (In preparation).

Project Title: Extrusion processing of skim milk proteins

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

Hal Johnson, Professor, Food Science and Nutrition, Brigham Young Univ., Provo, Utah 84602

Funding: Western Dairy Foods Research Center

Objectives:

Extrusion has been shown to offer great product versatility by using a thermodynamically efficient method for the cooking, texturization and forming of raw materials with varied functional characteristics. American consumers are becoming increasingly aware of the nutritional importance of proteins, vitamins and minerals, and are avoiding foods containing excess calories and saturated fats. Therefore, new products may require the addition of selected sources of proteins such as skim milk proteins, production of which is an established segment of the dairy industry. However, research specifically designed to modify skim milk protein to lend itself more readily to inclusion in new food products is lacking.

This project will determine operating parameters for extruding skim milk proteins to alter their characteristics so that they will be more widely used in fabricated food products. This research is a cooperative effort between faculty and graduate students at Utah State University and Brigham Young University. A blend of corn starch and skim milk proteins will be extruded. The control variables will be:

- (I) Moisture Content
- (II) Protein Content
- (III) Lactose/Protein
- (IV) Feed Rate

The extrudates will be analyzed according to the following response variables:

- (I) Expansion Ratio
- (II) Bulk Density
- (III) Viscosity-cooked and uncooked
- (IV) Color Change
- (V) Water Absorption Index
- (VI) Shear Force
- (VII) Product Temperature

These tests will be done by USU researchers. In addition, USU will also do Scanning Electron Microscopy on the extrudates. B.Y.U. will test the modification of covalent and ion-covalent interactions. Since these two separate sets of data will be collected on the same extrudates, careful coevaluation will result

in a better understanding of extruder processing parameters, their effect on structural modification and the corresponding change in functionality.

## RESULTS

Based on preliminary runs the ranges for the control variables were determined. To get protein rich skim milk powder, skim milk was ultrafiltered to 13.67%, 17.07%, and 19.88% total solids concentration. The skim milk at various concentrations was analyzed for protein and lactose content. It was then dried to get protein rich skim milk powder. To generate the experimental design, surface methodology was used. A statistical software package called ECHIP was acquired for this purpose. Using ECHIP, a central composite rotatable design was generated. This design is the most accurate design for a quadratic model. The aim is to express the response variables in terms of the control variables via a quadratic model. Once this is done, a response surface will be generated and the optimum conditions for extrusion will be identified. To get the product temperature, some modification was done on the extruder. A space i.e. an extra segment of barrel was added between the screw and the die and a rigid thermocouple was inserted: This insured that the temperature being read was the product temperature, not the barrel temperature.

## IMPACT OF RESEARCH

This research will enable us to determine the optimum extrusion conditions. Knowledge of the optimum extrusion conditions will lead to the development of new protein rich extruded products. Also, great potential lies in extruding meat-dairy protein. The results of this study could open the doors for the use of skim milk proteins in various kinds of extruded products. In fact, we are confident that this research will provide the foundation for future work in this area.

**Project Title:**

**Rheology and Microstructure of Mozzarella cheese**

**Personnel:**

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

Nabil Youssef, Dept. of Biology, USU.

Craig J. Oberg, Dept. of Microbiology, Weber State University

William McManus, Dept. of Biology, USU.

Robert Fife, Dept. of Nutrition and Food Sciences, USU.

**Funding:**

Western Center for Dairy Protein Research & 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 (including those already conducted at Utah State University), our knowledge of why Mozzarella cheese stretches and melts is still very limited. Part of this information will be provided in a study being funded by the National Dairy Promotion and Research Board on the development of culture systems and the use of fat substitutes for the manufacture of low fat Mozzarella. This project focuses on the development of experimental techniques and equipment to study the microstructure of Mozzarella cheese under melting conditions using microscopy.

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 specific objectives of this project are to:

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

**Results:**

The use of Environmental Scanning Electron Microscopy (ESEM) was investigated for its application in dynamically studying the melting and stretching of Mozzarella

cheese. Although this equipment allows for the examination of cheese without the sample dehydration necessary when using traditional SEM methods it does not provide the information on the interior structure of cheese. At the magnification available using (ESEM) the surface structure of the melted cheese was not very well resolved. As an alternative, work was conducted to increase the resolution of our existing SEM procedures.

A metal impregnation technique using tannin-ferrocyanide-osmium tetroxide to impart thermal conductivity to samples destined for scanning electron microscopy examination was adapted for milk products. 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) as well as the factory specified resolution of microscope (2.2 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.

#### **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. This also has application for the study of many other types of dairy products.

#### **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 Structure. (Submitted for publication)

**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 Dairy Foods Research Center and Utah Agricultural Experimental Station

**Objectives:**

During the last decade, yogurt was used as a base-material to manufacture desirable cultured dairy product, namely yogurt-cheese or Labneh. Yogurt-cheese "Labneh" has been defined as the product made from ordinary yogurt after part of the water, lactose and salts are removed.

We are developing a new, non-traditional, high protein non-fat dairy fermented product fortified with different flavors, vegetables, or fruits. This product may also be used as a new dairy ingredient for fortification of foods. The results of the proposed project will fulfill the requirements for priority three of the National Dairy Board for FY 1993 to find new or non-traditional products.

The main objective of this research project is to develop a process to use yogurt-cheese manufactured from skim 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 the different types of consumers according to age, sex, health, and national origin.

1. Develop high protein, non-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:**

Some preliminary experiments have been carried out to determine the best conditions to produce the base ingredient of high protein, low fat fermented dairy products. Also, some flavors have been tried. We are trying to adapt suitable conditions to get a product with high acceptability according to data from a sensory evaluation being done.

**Significance:**

This project will develop a method to manufacture from ultrafiltered skim and/or low fat milk a yogurt-cheese to produce dairy product 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.

**Publications:**

None

**Project Title:** Development of a process for production of UF milk retentate

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

Donald J. McMahon, Professor, Dept. of Nutrition and Food Science, Utah State University

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

**Funding:** Western Dairy Foods Research Center and Utah Agricultural Experimental Station

**Objectives:**

Several methods are available for processing surplus milk to extend its shelf life. Skim milk powder has been the standard means for storing surplus milk solids. Recently a frozen concentrate has also been developed. The major problem associated with frozen milk concentrate is the gradual destabilization of casein micelles that occurs during storage. Successful ultra-high temperature (UHT) concentrated milk has not yet been achieved because of the gelation that occurs in such concentrates upon storage of room temperature.

Production of milk powder by evaporative concentration and drying is an established segment of the dairy industry. However, product quality considerations favor the use of membrane separation to concentrate milk rather than thermal processes. Extensive heating during evaporative concentration often causes product degradation, primarily through change of color and flavors, and high denaturation of protein

Milk powder with better functional and nutritional quality can be produced employing membrane separation such as ultrafiltration for concentrating milk.

The primary objective of this project is to develop a process for production of high protein UF milk retentate powder by:

1. Determining effects of heat treatment, pH and limited enzyme treatment of UF retentate on the chemical, physical and functional properties of its resultant spray dried, high protein, milk powder.
2. Determining effects of drying parameters, such as particle size, air temperature, solid concentration and foam spray, on properties of the retentate powder.
3. Evaluating product applications of the high protein milk powder.

## Results:

Raw skim milk retentate with 20% solids produced by UF was subjected to different heat treatments and pH adjustments prior to spray-drying. The heat treatments were 65°C for 30 min, 75°C for 28 s, and 85°C for 28 s. pH was adjusted to 6.4, 6.7, and 7.0. Retentate powders were analyzed for moisture, protein, lactose, fat, ash, titratable acidity, and pH. Physical property determinations included solubility index, dispersibility, viscosity, scorched particles, poured density, packed density, and water absorption isotherm. No interaction effects of heat treatment and pH adjustment were observed. pH adjustments affected ash content and solution viscosity. Heat treatment affected solubility and poured density. pH adjustments and heat treatment had only minor effects on the measured properties.

Skim milk retentate powder with modified functional properties was produced using UF and spray drying processes. Pre-treatments of different heat treatments and pH adjustments were applied prior to spray drying. The heat treatments were 65°C for 30 min, 75°C for 28 s, and 85°C for 28 s. pH was adjusted to 6.4, 6.7, and 7.0. The functional properties of the retentate powders were determined. pH adjustments significantly affected gel water-holding capacity, acid gel strength, emulsifying capacity and foaming capacity. Heat treatment also significantly affected gel water-holding capacity, acid gel strength, and emulsifying capacity, but not foaming capacity. The interaction of heat treatment and pH adjustment affected gel water-holding capacity, acid-gel strength and emulsifying capacity.

Results showed that by using UF to concentrate raw skim milk to 20% total solids, high protein retentate powders with >60% protein can be produced which is nearly double the protein concentration of conventionally produced skim milk powder. Also, functional properties can be modified by applying heat treatments and pH adjustments prior to spray drying.

## Significance:

Results showed that high protein retentate powders with modified functional properties could be produced by using UF to concentrate skim milk as well as applying heat treatments and pH adjustment prior to spray drying. One more advantage for retentate powders compared with other milk powders is that the retentate powders permit desirable usage in many food systems such as yogurt, cheese, ice cream and bakery products at more economic transportation costs. This powder is used now in our trials to develop a fermented dairy product which will be used as a dairy base ingredient to produce new dairy foods.

**Publications:**

1. Bond, S. A. and C. L. Hansen. 1991. Whippability of enzyme treated skim milk retentate powder. J. Dairy Sci. 74 (suppl. 1):95.

2. El-Samragy, Y. A., C. L. Hansen, and D. J. McMahon. 1992. Production of ultrafiltered skim milk retentate powder. 1. Composition and physical properties. J. Dairy Sci. 76:388-392.

3. El-Samragy, Y. A., C. L. Hansen, and D. J. McMahon. 1992. Production of ultrafiltered skim milk retentate powder. 2. Functional properties. J. Dairy Sci. In Process.

4. Hansen, C. L., E. A. Ahmed and Y. A. El-Samragy. 1993. Whipped topping from enzyme-hydrolyzed skim milk retentate. J. Dairy Sci. 79 (Suppl. 1):141.

**Project Title:** Controlling Age Gelation of UHT milk Concentrates

**Personnel:** Donald J. McMahon  
Conly L. Hansen  
Mohamed A. Mohamed  
Nutrition & Food Sciences Dept., Utah State University

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

**Objective:**

Age gelation of ultra-high-temperature (UHT) milk concentrates has hindered the commercial use of milk concentration as a means of lowering transportation cost. Composition of milk, severity of heat treatment, sequence of operation, homogenization, use of additives, total solids, and enzyme treatment has been shown to affect age gelation of UHT milk. The specific objective of this project is to study the effects of the process parameters of UHT heating on age gelation of milk concentrates.

**Results:**

Eight UHT runs (four direct and four indirect) were completed. Two pre-heat temperatures (75°C, 90°C) with two holding times (20 s, 120s), and two sterilization temperatures (138°C, 145°C) with two holding times (4,16 s) were used in this study. Samples were stored at two temperatures (15°C, 35°C) for eight months. Changes in viscosity, sediment depth, browning, and pH were monitored at four weeks interval.

Samples stored at 15°C showed an abrupt increase in viscosity resulting in complete gelation of all samples. Sedimentation occurred in all samples, regardless of the storage temperature, and the sediment particles were undispersible. The sedimentation depth increased with storage time and severity of heat treatment. Browning was observed in samples stored at 35°C and to some extent at 15°C for samples receiving higher heat treatment. The

rate of pH reduction was greater for samples stored at 35°C than those stored at 15°C. From these results we concluded the following:

- 1- The shelf life of UHT milk concentrates was longer for samples processed using the indirect method (indirect heat exchangers) and it was longer for samples held for longer time at higher temperature.
- 2- Sedimentation depth was greater for samples processed by the direct steam injection method and was directly related to the severity of heat treatment and length of holding time as well as the storage temperature.
- 3- Samples stored at 15°C showed an increase in viscosity compared to those stored at 35°C.
- 4- The rate of reduction in pH was greater for samples stored at 35°C.
- 5- Browning was intense for samples processed at higher temperature, for longer holding time and processed by the indirect method. The extent of browning was greater at higher storage temperature.

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

Based on the results obtained from this study, we recommend the indirect plate heat exchanger as a method for UHT processing with respect to age gelation of concentrated milk. Preheating the milk before sterilizing is a critical step for extending the shelf life of UHT milk concentrates. The longer the holding time during preheat treatment and during sterilization the longer the shelf life of the UHT milk concentrate. Storage temperatures in the range of 10-15°C is recommended for a longer shelf life.

#### **Publications:**

Mohamed M. A. and D. J. McMahon. 1993. Influence of ultra-high temperature process parameters on age gelation of milk concentrate. *J. Dairy Sci.* 76 (Supplement 1):104.

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

**Personnel:** Donald J. McMahon  
M. Christopher Alleyne

Nutrition & Food Sciences Dept., Utah State University

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

**Objectives:**

1. To determine the influence of lactose concentration of milk concentrates on age gelation.
2. To determine the role of whey proteins (especially  $\beta$ -lactoglobulin) in the mechanism of age gelation of UHT sterilized milk concentrates.
3. To monitor changes in casein micelle structure that occur during storage of UHT sterilized milk concentrates and relate this to their stability

**Results:**

**Objective 1.** This has been completed.

**Objective 2 & 3.**

Using antibodies to  $\beta$ -lactoglobulin (J.J. Stastny, University of Illinois College of Medicine), Goat anti-mouse IgG conjugated to 10 nm gold (Ted Pella Inc.) and other electron microscopy and immunogold labeling materials a protocol for immunolabeling of milk samples was established and this will be applied to the other 5 milk proteins to determine their positions in fresh milk through UHT processing and storage until gelation. A tentative mechanism for concentrated UHT milk age gelation has been 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 0.5 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 properly the filled capsules. 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 microencapsulation but shows agar fibres in the micrographs of the milk samples. This is particularly undesirable when investigating, for example, intermicellar strands of gelled UHT milk concentrates. Microcube encapsulation produces superior quality images of the fluid food structure.

### **Abstract 2.**

Trichloroacetic acid precipitated milk whey protein from direct ultra-high temperature processed milk retentate (ultrafiltration concentrated 3x by volume reduction) was used to establish a protocol for aldehyde fixation and immunogold labeling of  $\beta$ -lactoglobulin in milk samples. Microcube encapsulation was the prefixation method for all samples. ELISA was used to investigate antigen degradation caused by aldehyde. Antigenicity of the samples was reduced by both aldehyde fixatives but paraformaldehyde was 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 0.5 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 preserved antigenicity while providing adequate fixation of the protein. The paraformaldehyde fixation resulted in better sections at labeling. LR White resin polymerized at 50°C was satisfactory for the embedding of samples. Teleosteam fish gelatin (0.1%) with normal goat serum (0.1%) in 20mM tris buffer without bovine serum albumin proved to be an



adequate blocking agent. The protocol was then used on gelled UHT and fresh UHT milk retentates to localize  $\beta$ -lactoglobulin.

### **Abstract 3**

$\beta$ -Lactoglobulin complexed with other milk proteins can be identified in dairy foods using immunolocalization techniques. Immunolocalization with embedding at 50°C in LR White resin was used to identify complexed  $\beta$ -lactoglobulin in eleven dairy products. The effect of the manufacturing process on the location and relative abundance of this whey protein was investigated. Heating temperature, duration of heating and pH during manufacturing affected the production of  $\beta$ -lactoglobulin complexes with other milk proteins. More stable  $\beta$ -lactoglobulin complexes were produced around an optimum temperature and low pH.  $\beta$ -Lactoglobulin complexed with caseins was more stable than  $\beta$ -lactoglobulin complexed with other whey proteins. The ripening process of cheese seemed to destabilize or remove  $\beta$ -lactoglobulin complex from the product. This immunolocalization technique can be used to identify  $\beta$ -lactoglobulin complex in other food systems and should prove useful in investigating fundamental manufacturing effects on the fate of this protein in foods.

### **Impact of Research:**

A mechanism of age gelation, elucidated through immunolocalization techniques and transmission electron microscopy, may be proposed.

### **Publications:**

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

Alleyne, M. C., W. McManus and D. J. McMahon. 1993. Comparison of antigenicity of  $\beta$ -lactoglobulin in UHT milk retentate and whey different modes of aldehyde fixation. *Food structure* (Approved for Submission)

Alleyne, M. C., W. McManus and D. J. McMahon. 1993. Immunolocalization of  $\beta$ -lactoglobulin in processed milk, yogurt and cheese samples. *Food structure* (Approved for Submission)

## WCDPRT PROGRESS REPORT

June 7, 1993

**Project Title:** Cloning the Nisin and Other Genes of Lactic Streptococci (lactococci) into *Leuconostoc* Species and Amplification of Nisin Production.

**Personnel:** W. E. Sandine, Dept. of Microbiology, Oregon State University  
H. A. Wyckoff, USDA/ARS, Peoria, IL 61604-3999  
Mary Barnes, 1404 Girard, San Marcos, TX 78666  
Kevin Gillies, Marschall Products, Madison, WI  
Marina Jovanovic, Graduate Research Assistant, Dept. of Microbiology, Oregon State University  
Jeff Broadbent, Dept. of Food Science, Utah State University, Logan, UT  
Jeff Kondo, Dept. of Food Science, Utah State University, Logan, UT

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

### Objectives:

1. To produce and characterize lactose positive *Leuconostoc* transconjugants obtained by conjugal matings between *Lactococcus lactis* and *Leuconostoc* spp.
2. To develop transformation and gene cloning systems in *Leuconostoc*.
3. To introduce into *Leuconostoc*, plasmid-coded-protease genes from lactic streptococci.
4. To use the genetically constructed fast acid-producing *Leuconostoc* to produce different fermented dairy products such as cultured buttermilk, cottage and Gouda cheese.
5. To conjugally transfer plasmid-coded nisin genes from *Lactococcus lactis* to *Leuconostoc*.
6. To amplify nisin production by gene cloning techniques.
7. To use nisin producing *Leuconostoc* in Swiss cheese manufacture to inhibit gas producing anaerobic spore formers such as *Clostridium tyrobutyricum*.
8. To study the inhibition of *L. monocytogenes* by nisin and to use genetic engineering to maximize its useful application.

## Results:

Results reported in this report are only those that have been obtained since the last progress report dated May 29, 1992. Using the transformation system developed and reported on in the last report and also published in the Journal of Dairy Science (74;1454, 1991), efforts were concentrated on introduction of lactococcal genes into *Leuconostoc*. This was facilitated by the isolation and characterization of a cryptic stable plasmid from *Enterococcus faecium* 226. It is a 3.8 Kb stable vector with 18 unique restriction sites. Sequence analysis of the minimal replicon identified a possible origin of replication and putative replication protein. Analysis showed that the vector was not of the single-stranded rolling circle replication type common to Gram positive bacteria. It was also found to be an extremely stable replicon with limited homology to the well-known lactococcal plasmid pCI305. Sequence analysis of the origin detected one open reading frame of 822 base pairs capable of encoding a 32 Kdalton protein corresponding to the results of an in vivo transcription/translation reaction. No detectable single-stranded intermediates were found for the replicon, suggesting that the cryptic plasmid, designated pMBB1, may be included in the same family as the widely used plasmid pCI305. Using the pMBB1, a small stably maintained vector containing a large multiple cloning region was constructed. This vector was successfully used in electroporating the  $\beta$ -galactosidase gene from *Streptococcus thermophilus* into *Leuconostoc* strains. High levels of the enzyme were detected in cell-free extracts of several *Leuconostoc* species which had either no enzyme or very low levels. This is the first recorded instance of transfer of genes from lactococci into *Leuconostoc* and while the  $\beta$ -galactosidase containing *Leuconostoc* were unable to ferment lactose, we now are coming more close to realizing this possibility. It is likely that the permease gene was not "transformed" along with structural  $\beta$ -galactosidase.

In other work related to the transfer of genes from lactococci to *Leuconostoc*, two *Lactococcus lactis* strains (LM2301 and LM2306) were found useful for insertional activation studies with the streptococcal transposon Tn919. In contrast to a previous report concerning *Lactococcus lactis* MG1363, we have found that Tn919 inserts into the chromosome of both LM 2301 and LM2306 in a multiple and random manner. This is significant in that it reports two possible candidates for insertional activation studies with Tn919 in *L. lactis*, which previously were

thought to be limited. Insertional activation of genetic information into the bacterial chromosome is a highly useful method to follow gene transfer and use of this transposon with the indicated strains will be useful in the future in following the introduction of lactococcal genes into *Leuconostoc*. Being able to do this will greatly improve the utility of *Leuconostoc* bacteria.

In the process of doing these genetic studies on the *Leuconostoc*, we were frustrated by the lack of a thorough study of the many *Leuconostoc* organisms that are in our culture collection. This was a hindrance to our work and so we embarked on a more thorough characterization of these bacteria in order to find strains that were suitable candidates for continued genetic researches. From our collection of over 100 strains, we did a thorough taxonomic study with the objective of determining for certain which strains were *Leuconostoc cremoris*. These are the ones that we could most easily identify to species and ones we are confident will prove useful in manufacturing dairy fermented products. About 20 strains of *Leuconostoc cremoris* have been thoroughly characterized to insure their identity and determine the variety of biochemical properties which they possess and these strains also are being analyzed for their ability to produce diacetyl in association with lactococci. They also are being analyzed for bacteriocin-producing capabilities, since strains producing these inhibitory substances would not be good candidates for use in fermented dairy products since they likely would inhibit the lactococcal strains with which they were grown. Several *Leuconostoc* have been found which are inhibitory for lactococci and this emphasizes again the need to be able to construct *Leuconostoc* strains with protease and  $\beta$ -galactosidase activities so that they can be used as single cultures in the manufacture of fermented dairy products.

Objectives 5 through 8 of this research project were studied by Dr. Kondo and Dr., Broadbent at Utah State University. Their most recent progress has been communicated in the form of an abstract of a paper to be presented at the 1993 meetings of the American Dairy Science Association at the University of Maryland, June 13 - 16. The abstract of that paper follows:

"Conjugation was used to transfer genes associated with nisin production from nisin- and sucrose-positive (Nip<sup>+</sup>Suc<sup>+</sup>) *Lactococcus lactis* subsp. *lactis* donors to sucrose-negative (Suc<sup>-</sup>) *Streptococcus salivarius* subsp. *thermophilus*

recipients. Streptococcal recipients were constructed by electro-transformation with the erythromycin-resistance plasmid pGK13, or by conjugal introduction of the enterococcal plasmid pAM $\beta$ 1. Matings between lactococcal Nip<sup>+</sup>Suc<sup>+</sup> donors and streptococcal recipients that contained pAM $\beta$ 1 yielded Suc<sup>+</sup> transconjugants that were immune to 7 mg per ml of nisin but which did not produce the bacteriocin. Genetic and phenotypic analysis of these transconjugants demonstrated that they had acquired the nisin structural gene, *nisA*, as well as other genes encoded by the lactococcal nisin-sucrose conjugative transposon, Tn5301. Finally, transconjugants were not detected in matings with recipients that contained pGK13, which suggested that pAM $\beta$ 1 may have facilitated intergeneric transfer of nisin genes."

### Impact of Research

Having demonstrated ability to transfer genes from lactococci into *Leuconostoc*, it may be expected that future studies with this system will enable us to construct strains which will be improved for use in dairy fermentations. *Leuconostoc* able to ferment lactose and degrade milk protein will be highly useful in the manufacture of more uniform dairy products and also for use in the production of new specialty dairy products.

The work with nisin had the ultimate objective of being able to construct super nisin producing strains for use in food preservation. While transfer of the nisin genes has been accomplished, continued research is necessary in order to clone those genes into stable vectors which can be used to amplify the nisin production. Such an accomplishment would be highly significant in terms of producing large amounts of nisin for use by the dairy industry.

## **Publications:**

Broadbent, J. R., J. K. Kondo and W. E. Sandine. Intergeneric transfer and Expression of Genes Associated with Nisin Production. *Journal of Dairy Science*, 76(supplement 1):122, 1993.

Barnes, M., K. Gillies, and W. E. Sandine. 1993. Transposon Mediated Gene Insertions into Chromosomal and Plasmid DNA of Lactococci. *Food Microbiology*, In Press.

Wyckoff, H. A. 1992. Development and Use of Genetic Techniques for Study of Dairy *Leuconostoc* bacteria. Ph.D. Thesis, Oregon State University, 94 pages.

Wyckoff, H. A. 1993. The Dairy *Leuconostoc* : an overview of their molecular genetics. *J. Dairy Sci.* 76(supplement 1):133 (abstract).

## WCDPRT PROGRESS REPORT

June 10, 1993

**Project Title:** Growth of Bifidobacteria in Milk: Association with *Streptococcus thermophilus* and *Lactobacillus* species as measured by genetic and enzyme probes.

**Personnel:** Joseph W. Booth, Dept. of Biochemistry and Biophysics  
Oregon State University  
Janine E. Trempy, Dept. of Microbiology, Oregon State  
University  
William E. Sandine, Dept. of Microbiology, Oregon State  
University

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

### Objectives:

1. Development of an antibody against fructose-6-phosphate-phosphoketolase (F6PPK). Fructose-6-phosphate-phosphoketolase is an enzyme unique to bifidobacteria. Antibody production necessitates purification of the enzyme in quantities sufficient for injection into rabbits. With the antibody in hand, Western blots of colony lifts of plated cultures from fermented dairy products can be performed.
2. Development of a genomic probe against the gene for fructose-6-phosphate-phosphoketolase. Achievement of a homologous genomic probe necessitates the cloning of the gene for fructose-6-phosphate-phosphoketolase. *E. coli* cells containing the cloned gene can be identified from a library of cloned sequences by screening the library with the antifruuctose-6-phosphate-phosphoketolase antibody.

## Results:

In the last report we described the purification to homogeneity of the enzyme fructose-6-phosphate-phosphoketolase, a protein exclusively found in bifidobacteria. Since that time experiments to optimize growth conditions for the bifidobacteria to maximize enzyme production and stability have been conducted and the best medium found to date for growing the organisms for enzyme extraction has been determined to be half strength PMN broth. Half strength PMN broth contains, per liter; 25 g of Peptonized Milk Nutrient obtained from Sheffield Products, a division of Quest International, in Norwich N.Y., 20 g of primaton meat extract also obtained from Sheffield Products, 1.0 g of yeast extract, 5 g of lactose and 1.0 ml of Tween 80.

Originally it was intended to use the rabbit prepared antisera for developing of an enzyme linked immunosorbant assay (ELISA) but it since has been decided to use monoclonal antibody. Oregon State University has an operational monoclonal antibody facility and leukocytes from immunized responsive mice have been collected and fused with a mouse myeloma cells. Residual spleen cells have been frozen for subsequent fusions as well. At the present time spent medium from hybridoma cultures are being tested for the presence of antibody that binds to the fructose-6-phosphate-phosphoketolase. Positive cells will be grown in expanded cultures and frozen for subsequent use in developing the ELISA assay. Prior to preparing the monoclonal antibody, a nitrocellulose colony lift procedure was developed for establishing the colony matrix upon which the ELISA tests will be conducted.

The presently used purification procedure of the fructose-6-phosphate-phosphoketolase involves essentially four main steps: 1) Bead beating for extract production; 2) protamine sulfate precipitation for removal of the nucleic acid; 3) Sephacryl S-300 gel filtration; 4) Mono-Q anion exchange chromatography using fast pressure liquid chromatography. Beginning with crude extract that has about 5 units of activity per mg of protein, the purified material has a specific activity of well over 100 units per mg of protein. Percentage recovery of activity units of enzyme in the crude extract is arbitrarily established at 100 and in the purified material approximately 40% of those units are recovered.



Experiments are now in progress to characterize the enzyme thoroughly with respect to optimum temperature, optimum pH, kinetic properties, nature of active site as revealed by inhibitor studies and stability characteristics. Also in progress is construction of a genomic library from *Bifidobacterium brevis* from which the gene for F6PPK will be cloned. With the cloned gene in hand, the sequence for F6PPK can be obtained and a gene probe based on those sequences can be constructed. The gene probe will be used to hybridize to colony lifts of plated bifidobacterial-containing yogurt cultures to provide an alternative method of quantitating viable bifidobacterial cells in dairy products.

### **Impact of Research:**

Sweet Acidophilus milk has represented a significant product for the dairy industry in terms of volume consumed. The same may be said of yogurt, some of which now reveal on the label the presence of *Lactobacillus acidophilus* and even bifidobacteria. Bifidobacteria are promoted in Japan and also in Europe as bacteria which produce unique health benefits including elevation of immunocompetence, and reduction in the incidence of colon tumors. Data in the literature from well controlled scientific experiments support these conclusions (see review by Sanders entitled "The effect of lactic cultures on human health", Advances in Food Nutrition Research, In Press). Furthermore, research sponsored by the California Dairy Foundation is now underway to develop the next generation dairy product of this type. It will contain *Lactobacillus acidophilus*, *Bifidobacterium* species, and *Streptococcus thermophilus*. This product will offer advantages to those who suffer from lactose malabsorption and deliver strains of lactobacilli and bifidobacteria which will adhere to the intestinal tract. To produce uniformly high quality products containing bifidobacteria requires a simple method of accurately enumerating the number of those types of bacteria present. While many selective media have been described for use in enumerating bifidobacteria, each suffers from limitations such as lack of selectivity and suitability, only for certain strains. Therefore, the ELISA and gene probes are justified on the basis of providing a sensitive and accurate method for determining exact numbers of bifidobacteria present in fermented products containing these bacteria. This will allow establishment of minimum standards of viable bifidobacteria as now exists for numbers of lactobacilli in Sweet Acidophilus milk ( $2 \times 10^6$  per ml). This way the customer will be assured of

consuming products which contain an appropriate minimum number of bifidobacteria at the time of consumption. Use of the selective method based on the ELISA technique also will allow future studies to examine strain and species variability among the bifidobacteria to select those that are the most desirable for use in fermented dairy products. There is little doubt that the future will see more and more use of bifidobacteria in fermented products and the technologies offered by this research will facilitate that.

**Publications:**

None.

**Project Title:**      **Characterization of Milk Proteolysis by Lactococcal Starter Culture Strains Using Amino Acid Analysis**

**Personnel:**        Rodney J. Brown  
                         Christina Beer  
                         Nutrition & Food Sciences, Dept., Utah State University

**Funding:**            WESTERN DAIRY FOODS RESEARCH CENTER

**Objectives:**

1.      To genetically construct lactococcal strains with different proteolytic (PrtP and PrtT) and lactose utilizing capabilities.
2.      To use amino acid analysis to study the interactions of milk proteolysis genes (PrtP and PrtT) of the genetically constructed strains.
3.      To characterize growth and acid production of the genetically constructed strains and determine the effects that maintenance of plasmids have on growth and acid production.
4.      To examine the effect of different expression levels of proteinase genes (PrtP and PrtT) to try and improve flavor and texture characteristics in cheese, and examine the potential of these results for application to accelerated cheese ripening.

**Results:**

**Objective 1:**

    This part of the research has been completed.

**Objective 2:**

    Amino acid analysis has been done on the strains when grown in whole pasteurized milk. The proteolysis of individual caseins is in progress and will be completed at the end of August.

**Objective 3:**

    Characterization of growth and acid production has been completed. The characterization of plasmid maintenance will begin in August.

**Objective 4:**

    This objective has not been completed yet.

### **Impact of Research:**

Proteolysis from bacterial starter cultures play a significant role in the physical and organoleptic properties of cheese and other fermented dairy products. Improper proteolysis can result in a wide number of defects, including bitterness, texture, and body problems. We have ways to measure gross proteolysis but are very limited in techniques to profile or characterize proteolysis for individual bacterial strains. Understanding of the specific action of proteinases and peptidases upon specific milk proteins and a correlation of that activity to specific physical and organoleptic properties would be of great value to the dairy industry. This project has the potential to greatly enhance product quality, allow for the production of products with enhanced properties, and even allow the development of new products by using bacterial strains with different proteolytic abilities. This method would also be very valuable in identifying and characterizing newly developed strains for biotechnological endeavors.

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

**Personnel:** Bruce Geller, Assistant Professor, Department of Microbiology, Oregon State University.

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

**Objectives:**

1. Make *in vitro* mutations in the cloned gene required for phage infection.
2. Insert the mutated copies of *pip* into the chromosome and exchange it with the wild-type gene.
3. Test the different mutants for viability, phage resistance, and growth characteristics favorable to cheese-making.

**Results:**

We have screened phage m13-resistant mutants of *Lactococcus lactis* subsp. *lactis* ML3, and found some that are complemented by pBG1. This confirms that *pip* is required for phage infections in more than one strain of *Lactococcus lactis* subsp. *lactis*. In addition, the phage m13-resistant mutants were resistant to 11 other phages, but were complemented to the phage-sensitive phenotype when transformed with *pip*. This suggests that *pip* may be required for many, and perhaps most phage infections in strain C2 and ML3. We are currently screening phage C2-resistant mutants in *Lactococcus lactis* subsp. *cremoris* KH to determine if *pip* is required in other subspecies.

We have begun to sequence the mutated copy of *pip* from the spontaneous phage c2-resistant strain of *Lactococcus lactis* subsp. *lactis* C@, that we named RMC2/4. We have determined that *pip* is fully contained on a unique, 12 kb XbaI fragment of the chromosome from *Lactococcus lactis* subsp. *lactis* C2. Knowing this we made a genomic library of XbaI digested chromosomal DNA from RMC2/4. We are in the process of screening *E coli* clones for mutated *pip*, using colony hybridization with a labeled wild-type *pip* as a probe.

We have initiated studies to determine if *pip* is required for viability. To do this we will exchange, by homologous recombination, the wild-type chromosomal copy of *pip* for a Tn5-mutated copy of *pip*. The Tn5-mutated copy of *pip* was constructed in the course of our investigation of the wild-type *pip* (Geller *et al.* 1933, J. Bacteriol., in press). We experienced a technical difficulty in sub-cloning Tn5-*pip* into an appropriate integration vector, in that the XbaI fragment containing Tn5-*pip* in pSA3 was exactly the same size as XbaI-restricted pSA3. Therefore, we could not separate the two fragments by electrophoresis. We solved that problem by finding a restriction site (KpnI) on pSA3 that is not present on the XbaI fragment of Tn5-*pip* from pSA3. This allowed us to reduce the size of the XbaI fragment of pSA3, separate the Tn5-*pip* XbaI fragment by electrophoresis, and sub-clone the latter into a modified pACYC184. We found it necessary to modify pACYC184 by replacing the tetracycline resistance gene with the erythromycin resistance gene (from pSA3), in order to have a selectable

marker in *Lactococcus*. Currently we are attempting to select chromosomal integrants of the Tn5-*pip*.

**Impact of research:**

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.

This 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.

**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  $\beta$ -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  $\beta$ -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:

Project objectives 1 through 3 were completed during the first and second years of the project. The adsorption kinetics exhibited by  $\alpha$ -lactalbumin ( $\alpha$ -lac),  $\beta$ -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  $\beta$ -lactoglobulin ( $\beta$ -lg) adsorption kinetics on silicon surfaces varying in wettability. With regard to  $\beta$ -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,  $\alpha$ -lac,  $\beta$ -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  $\alpha$ -lac,  $\beta$ -casein,  $\beta$ -lg and BSA interfacial behavior at air-water interfaces, and  $\alpha$ -lac,  $\beta$ -lg and BSA interfacial behavior at solid-water interfaces.  $\beta$ -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  $\alpha$ -lac and BSA behaved in a manner more similar to that of  $\beta$ -lg at each surface; however, the difference in  $\alpha$ -lac adsorption to hydrophilic and hydrophobic surfaces was quite large, with  $\alpha$ -lac adsorbing to a much greater extent on hydrophobic surfaces.  $\alpha$ -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  $\alpha$ -lac in the same time period. 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 Image analysis was used for visualizing and enumerating bacterial cells adhered to surfaces. Initial results clearly indicated significant differences in the numbers of adhered cells to surfaces with different adsorbed proteins. Most dramatic were BSA treated surfaces which had 4-5 fold less adhered bacteria as compared to the non-globular proteins.

### Impact of Research:

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:

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. Surface hydrophobic influences on  $\beta$ -lactoglobulin adsorption kinetics. *J. Colloid Interface Sci.*, 154:337

Suttiprasit, P., Krisdhasima, V. and McGuire, J. The surface activity of  $\alpha$ -lactalbumin,  $\beta$ -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. The surface activity of  $\alpha$ -lactalbumin,  $\beta$ -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:** Interactions between milk proteins, starter cultures and hydrocolloidal milk fat replacers

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

Don McMahon, Dept. of Nutrition and Food Sciences,  
Utah State University

Mike LaFevre, Dept. of Nutrition and Food Sciences,  
Utah State University

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

**Objectives:**

In response to consumer demand, hydrocolloid fat replacers have been developed by many food companies. Some of fat mimetics may have properties that are useful to replace milk fat in cheese products. We hypothesize that fat replacers interact with milk proteins and dairy starter cultures that aid in developing fat-like characteristics to lowfat dairy products. To test this hypothesis, we will study interactions of milk protein, dairy starter cultures and milk fat replacers that are claimed to have milk fat mimetic properties. The specific objectives of this research are to:

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

**Results:**

Initial microbial characterization was done using three commercial starter cultures used for in production of 33% reduced fat Cheddar cheese. Studies to expand the number of useful starter cultures for lowfat cheese manufacture have been conducted by characterizing 45 other strains in the USU culture bank. Each strain metabolizes each fat replacer differently. Growth and acid production increase when starch-based fat replacers are added to milk and M17 media. However, protein-based fat replacers inhibit growth and slow acid production significantly.

A rapid semi-automated screening assay was developed to monitor glucosidase activity in starter cultures to aid in selecting useful cultures for use in lowfat cheese. Glucosidase activity is strain dependent and predicts the ability of starters to utilize

starch-based fat replacers. Additional culture characterization and identification was done using the Biolog system.

Lowfat cheese (50% reduced) has been made using different single starter strains in combination with each fat replacer. Each strain and fat replacer contributes different flavors to the final product. Control of acid production in the vat was difficult with starch-based fat replacers, as was predicted from the culture screening data.

**Impact of Research:**

Manufacture of lowfat cheese is difficult because limited starter strains are available that produce commercially acceptable cheese. Little information is available concerning use of fat replacers and their interaction with starter cultures. This research points out that strain selection for manufacture of lowfat cheese is critical for production of acceptable cheese.

**Publications:**

LeFevre, M., and B. Weimer. 1993. ADSA, College Park, Maryland.

**Project Title:** Production of extracellular proteases of *Brevibacterium linens* for use in lowfat cheese

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

Ben Dias, Dept. of Nutrition and Food Sciences,  
Utah State University

Soni Ummadi, Dept. of Nutrition and Food Sciences,  
Utah State University

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

**Objectives:**

*B. linens* has been established to produce a variety of serine proteases during different phases of growth depending on environmental conditions. We hypothesize that these organisms will be useful to produce lowfat cheese with acceptable flavor, body, and texture based on the action of these proteases. This hypothesis will be tested by conducting the following specific objectives:

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

**Results:**

Nine strains of *B. linens* have been screened for growth, aminopeptidase (AP), protease, lipase, and glucosidase activity with and without starch and protein-based fat replacers. All screened activities are strain and fat replacer dependent.

Maximum growth occurred between 36 and 48 h and remained in stationary phase for at least 96 h, when testing was terminated. Specific growth rates changed with milk fat and fat replacer concentration. Higher concentrations of fat replacers slowed the growth rate, while at lower concentrations fat replacers enhanced the growth rate of all strains.

Cellular location for different AP activities were determined with the exocellular and intracellular fractions demonstrating the highest activity. Intracellular activity varied with each strain tested. Strain 8377 contained the highest total activity while strain 9175 contained the least and was almost exclusively valine AP activity. Exocellular AP activity was high and all strains contained moderate arginine and lysine AP activity. No strains contained measurable proline or arginine carboxypeptidase activity. Total AP activity was comparable to that of *Lactobacillus casei* and *Lactobacillus helveticus* strains used in lowfat cheese manufacture commercially.

Two strains showed the highest extracellular protease activity which developed between 48 and 60 h of growth. The activity increased to 96 h when the testing was terminated. Studies are continuing to determine the effect of fat replacers on the activity of extracellular proteases.

Lipase activity was inversely related to the fatty acid chain length tested. This characteristic was strain dependent with 5/9 strains showing high total activity. Butyrate lipase activity was the highest in many strains. Stearate activity was the lowest or not detectable in all strains.

Glucosidase activity was strain dependent. Three strains contained  $\alpha$ - and  $\beta$ -galactosidase activity, suggesting that these strains can grow in milk via sugar metabolism. The other strains contained little or no glucosidase activity, indicating that they must use amino acids as the carbon source in milk if starch-based fat replacers are not added.

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

These data have provided information about the metabolic characteristics of *B. linens* that impact lowfat cheese flavor. Based on these data strain selection of *B. linens* for use in lowfat cheese production is critical to achieve an acceptable finished product. The strain selected for initial cheese trials produced an acceptable flavor at 1 mon, but at 2 mon the flavor was too bitter and rancid. Small scale cheese trials are continuing using different strain combinations to produce a balanced, aged 50% reduced Cheddar cheese.

#### **Publications:**

**OPERATIONAL ADVISORY COMMITTEE  
BUSINESS MEETING AGENDA**

1. Acceptance of 1992 OAC Meeting Minutes
2. JANET C. WILLIAMS (NDPRB) Presentation
3. WCDPRT Financial Report
  - A. Summary of currently funded projects
  - B. Budget activity for FY94
4. New (1993 to 1994) WCDPRT Research Proposals
5. NDPRB Competitive Research Proposals Approved
6. Review of low-fat cheese research
7. Set Date for WCDPRT 1994 OAC Meeting (OSU)
8. Other Business - new director for WCDPRT
9. Adjournment

## 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 1993-95  
(continued)**

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</i> <i>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
<hr/>				
TOTAL	\$329,232	\$454,064	\$154,513	\$68,026

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

**Projected Spending  
Fiscal Year 1994**

**[INSTITUTIONAL SUPPORT** **[\$959,180]**

**NDPRB CONTRIBUTION** **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 Assoc.	10,000
Kraft-General Foods, Inc.	5,000
Schreiber Foods, Inc.	5,000
Marschal-Rhone Poulenc, Inc.	5,000
USDA-ARS	200,000
State of Utah Center of Excellence "Center for Dairy Foods Technology"	187,775

**SUBTOTAL DAIRY RESEARCH CONTRIBUTIONS** **\$1,052,775**

**CARRY-OVER FUNDS TO FY94** **133,924**

**TOTAL AVAILABLE FUNDS FOR FY94 RESEARCH** **\$1,186,699**

**FY94 RESEARCH FUNDING:**

	NDPRB	MATCH
Research Area 1. Fundamental research (WCDPRT)	\$100,000	\$179,713
Research Area 2. Practical research (WCDPRT)	100,000	143,697
Research Area 3. Low-fat cheese research program	275,000	25,000
WCDPRT Administrative	25,000	25,000
Directed research at University of Idaho (supported by United Dairymen of Idaho matching funds)	-----	19,438
USDA-ARS	-----	166,076
State of Utah Center of Excellence "Center for Dairy Foods Technology"	-----	127,775

**TOTAL FY94 RESEARCH FUNDING** **\$500,000** **\$686,699**