EFFECT OF DENSE PHASE CARBON DIOXIDE ON ENZYME ACTIVITY AND CASEIN PROTEINS IN RAW MILK

A Thesis

Presented to the Faculty of the Graduate School

Of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Masters of Science

by

David Aldo Tisi

January 2004

ABSTRACT

Non-thermal methods of reducing bacterial numbers have been studied for more than a century. A majority of this work has focused on high pressure processing (>100 MPa). The addition of carbon dioxide (CO_2) at lower pressures (<100 MPa) is a relatively new process, but has demonstrated similar bactericidal effects. Pressurized CO_2 has also been shown to inactivate enzymes, but this ability has not been studied in milk. Dense phase CO_2 is defined as CO_2 which exists in the liquid or supercritical state. A proprietary piece of equipment designed by Praxair (Chicago, IL) applied dense phase carbon dioxide to milk in a continuous manner. Enzyme activity was assayed by acid degree value and protein breakdown. Temperatures between 15°C and 40°C and pressures between 7 MPa and 62 MPa were investigated. Because of milk's susceptibility to pH change, the process's effect on casein proteins was also studied. At higher temperatures (40°) and CO_2 concentrations, the process was shown to significantly reduce the proteolytic and lipolytic endproducts. However, these conditions also changed the structure of the underlying casein protein. By reducing the severity of treatment both in terms of temperature and CO_2 content, no change to the casein was observed. However, in these less severe conditions no significant effect on the rate of lipolysis or proteolysis was observed.

BIOGRAPHICAL SKETCH

David was born in Euclid, Ohio on Friday March 16th, 1979 to Aldo and Carolyn Tisi. After learning about Food Science from his aunt, Margaret Gerwin, he applied and was admitted to Cornell University in 1997. While attending Cornell, David had the opportunity to intern at several companies including Nestle and Pepsico. After 4 years of rigorous undergraduate study, David enrolled as a Masters student under the tutelage of Professor Joseph Hotchkiss.

While attending Cornell, David was extremely involved in extracurricular activities. Marching band, Model United Nations, College Bowl, and Product Development Team were among his pastimes. In later years, David parlayed his involvement with the IFT to become chair of the College Bowl Competition and Area Representative to the North Atlantic region.

David completed his graduate work on dense phase carbon dioxide and graduated in January of 2004. In the future David plans on entering the food industry in some capacity; and hopes that he leaves this world is a slightly better position than which he found it.

Dedicated to my parents,

Aldo and Carolyn Tisi

for their love, support, guidance and encouragement

ACKNOWLEDEMENENTS

Thank you Dr. Hotchkiss for giving me the opportunity to complete a Masters, here at Cornell. I have grown much in the time I have spent here as a graduate student and ultimately have you to thank.

Professor Barbano, you have the greatest work ethic I have ever seen, and I strive to approach the example you have set. You were under no obligation to help with my experiments though you volunteered your time and equipment to help me complete my charge. Thank you. I also want to thank your staff who were diligent in their assistance to my analytical work. Thank you Bob, Maureen, Pat, Laura, and Joanna for all your support.

Professor Thomas, I thoroughly enjoyed logistics as a minor. Not a day goes by that I can't find something that would run better through aggregation.

Mom and Babbo, I thank you most of all. You've been there to catch me when I fall; pick up my pieces when I fail; lend a high-five when I succeed; and plant a kick in the ass when I "do something stupid". Most importantly, a warm hug throughout.

Mary and Becky, although the lessons you taught me were quite different in nature, they were both invaluable nonetheless – Best regards.

To the rest of the department, my friends in the lab, and hundreds of brilliant Cornell undergrads and grads. You've all helped me out, and I thank you. Oh yeah, and Jesus... that part for dying on the cross, and turning yourself into crackers… thanks; that was sweet.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

INTRODUCTION

1.1 Non-thermal Processes

The concept of non-thermal processing of foods has been around for some time. In one of the earliest experiments with non-thermal processing of food, Hite (1899) demonstrated the bactericidal effect of high pressure processing (HPP). He reported that exposing milk to 600MPa of pressure for one hour at ambient temperature was able to extend the quality of milk by four days. Hite effectively did what most researchers hope to accomplish with HPP; destroy the endogenous bacterial population of a material without compromising some of its more delicate properties. In food products these include texture, flavor, vitamin activity, and enzymatic characteristics.

HPP (pressures above 100 MPa) has been studied intensively for a number of years and continues to the present (Yuste, Capellas et al. 2001). It is currently being used commercially to process higher value products that are perishable and have few processing alternatives such as guacamole and salsas. Although the FDA has not issued a final ruling, an additional benefit of using pressure to preserve foods might be to label foods as “fresh”, a term highly valued by consumers.

A proposed alternative to HPP is a system that utilizes carbon dioxide (CO_2) at lower pressures to inactivate bacteria (Kamihira, Taniguchi et al. 1987). While the pressures typically used in pressurized CO_2 processing approach 70 MPa, they are considerably less than the >600 MPa commonly used in HPP (Yuste, Capellas et al. 2001). Lower pressures would entail

smaller vessels, require less pumping power, and be more adaptable to continuous processing.

1.2 Dense Phase Carbon Dioxide

As indicated by the phase diagram (figure 1) CO_2 can readily exist in several states of matter with different characteristics. The term “dense phase” (DP)* as used here denotes those phases of matter that remain fluid, yet are dense with respect to gaseous CO_2. Consequently, this refers to the liquid and supercritical (SC) regions of the CO_2 phase diagram. At these states, CO_2 alters its physical properties by increasing its density, becoming a more effective solvent. In addition to the increased density in the liquid state, SC CO_2 also exhibits decreased viscosity and increased diffusivity (Rizvi, Daniels et al. 1986), which should facilitate penetration through a bacterial cell membrane. Experiments which used CO_2 but not necessarily under DP conditions as defined above are referred to as high pressure (HP) CO_2 processing.

While other gasses (N_2O, N_2, Ar, tetrafluoroethane) have been evaluated for bactericidal efficacy at pressures higher that atmospheric, only N_2O and CO_2 have demonstrated bactericidal effects (Balaban, Arreola et al. 1991; Wei, Balaban et al. 1991; Nakamura, Enomoto et al. 1994). The superior effect CO_2 has over other gases at these temperature pressures has been attributed to its low critical point: 7.11 MPa, 31ºC (table 1)

---

* SC CO_2 Processing: use of CO_2 in the SC phase only
DP CO_2 Processing: any use of CO_2 in the SC and liquid states
HP CO_2 Processing: any use of CO_2 above atmospheric pressure
CO_2 Processing: any use of CO_2

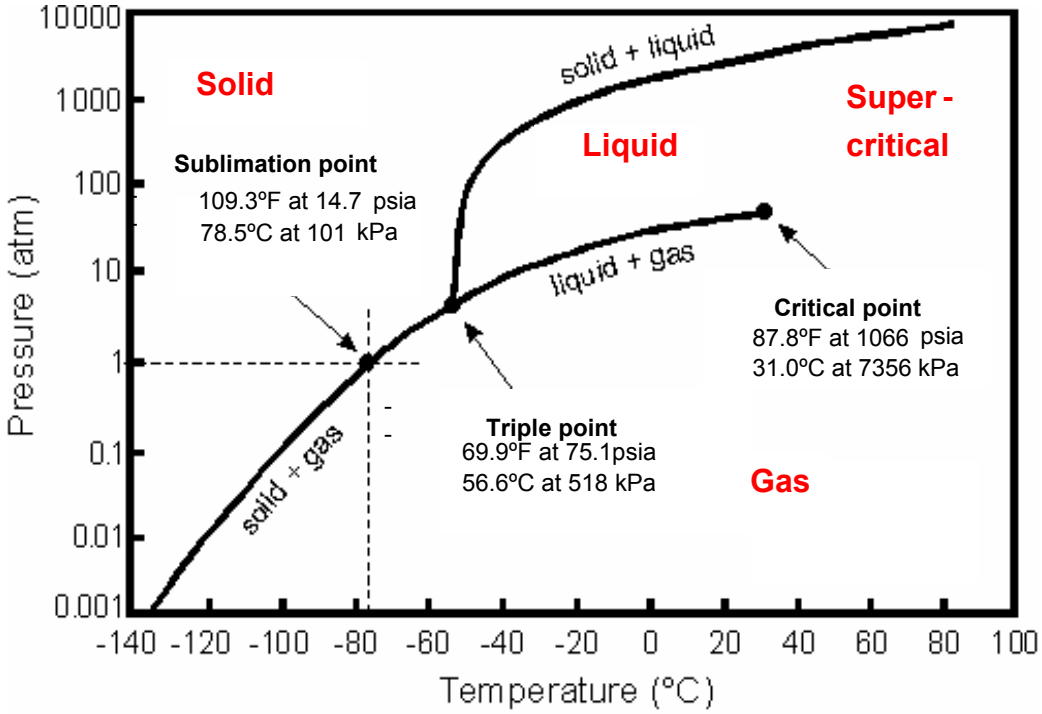

Figure 1

Pressure – temperature phase diagram of carbon dioxide

(Dillow, Dehghani et al. 1999). It is supposed that the increased diffusivity of CO_2 at supercritical conditions facilitates intercellular carbonation, leading to cell death.

Though N_2O has been effective at killing bacteria, CO_2 remains the most preferable gas for killing food organisms because of its low toxicity, nonflamability, and low cost (Enomoto, Nakamura et al. 1997). Furthermore, as an additive, CO_2 would not negatively effect a consumer's perception because of their familiarity with products such as carbonated beverages.

Table 1 Properties of some supercritical fluids at the critical point (Rizvi 1986)

Fluid	Critical Temp. (ºC)	Critical Pressure (MPa)	Critical Density (g/ml)
CO_2	31.0	7.11	0.47
N_2O	36.5	7.1	0.45
Water	374.2	21.5	0.32

1.2.1 Previous Uses of CO_2 Processing

The ability of CO_2 to inhibit the growth of bacteria at near atmospheric pressures has been known for some time (King and Nagel 1967). More recently, it has been found that if CO_2 is pressurized, the process is no longer bacteriostatic, but bactericidal (Arreola, Balaban et al. 1991; Nakamura, Enomoto et al. 1994; Enomoto, Nakamura et al. 1997; Erkmen 2000; Erkmen 2000; Erkmen 2000; Calvo and Balcones 2001; Erkmen 2001).

As with any technique, the use of HP CO_2 to kill bacteria has been tested on a wide range of materials to determine which usage would be most viable. Early work on bacteria focused on medical applications where thermal

treatment of sensitive materials or medicines could damage the item being sterilized (Kamihira, Taniguchi et al. 1987).

HP CO_2 processing's first use in food showed effective bacterial cell reduction in a variety of products including mozzarella, Parmesan, strawberries, onions, and fresh herbs (Haas, Prescott et al. 1989). Murat Balaban's research group at the University of Florida used the technology on liquid food products including orange juice and whole egg (Wei, Balaban et al. 1991). Work on juice has subsequently been carried on by other investigators who conducted research on several varieties (Hill, Marshall et al. 2000; Park, Lee et al. 2002).

More recently, HP CO_2 has been used to kill microorganisms (Calvo and Balcones 2001; Erkmen 2001; Erkmen 2001; Erkmen and Karaman 2001) and precipitate casein proteins (Jordan, Lay et al. 1987; Tomasula, Craig et al. 1995; Tomasula, Craig et al. 1997; Tomasula, Craig et al. 1998; Hofland, van Es et al. 1999) from milk.

1.3 Effect on Microbiology

The degree of bacterial kill from the application of CO_2 varies widely between treatments. Much of this inconsistency is due to the many parameters that can be altered during experimentation. Typical variables include CO_2 pressure, temperature, time, substrate, bacteria type, reactor type (continuous or batch), and number of pressure cycles.

Kamihira et al (1987) showed a 4-6 log reduction in the number of *E. coli* after treating an aqueous suspension with HP CO_2 for 2 hours at 20°-35ºC and 4-20 MPa[†]. Baker's yeast under the same treatment conditions showed

[†] 1 MPa = 9.87 atmospheres = 145 pounds per square inch

less than one log reduction. The theory that yeast cells are more resistant has been contested in more recent literature (Wei, Balaban et al. 1991; Arroyo, Sanz et al. 1997; Enomoto, Nakamura et al. 1997). However, Kamihira (1987) reaches the same conclusion as later studies that indicate both bacteria and yeast cells require appreciable water content for pressurized CO_2 inactivation (Haas, Prescott et al. 1989; Nakamura, Enomoto et al. 1994; Kumagai, Hata et al. 1997).

Perhaps the first individuals to apply HP CO_2 to food were a group working at General Foods (Haas, Prescott et al. 1989). They succeeded in destroying bacteria in a range of products from cheese to fresh herbs. Pressures between 2 and 6 MPa and times in excess of 2 hours reduced bacterial counts between 2 and 5 logs. Higher pressures and temperatures increased the effectiveness of the treatment.

(Arreola, Balaban et al. 1991) used SC CO_2 on abused orange juice samples to determine effect on total plate count. Pressures were between 8.3 and 33.1 MPa at temperatures between 35˚C and 60˚C in a batch system. The highest temperature and pressure treatment returned the lowest D value observed (12.7 minutes). Because endogenous microorganisms in orange juice live in an acidic environment they may be more tolerant to low pH than organisms in milk. If so, they would be more resistant to the acidifying effects of CO_2 indicating that DP CO_2 treatment may be more lethal to the native population of bacteria in milk.

Nakamura et al (1994) observed an 8 log reduction in the number of *Saccharomyces cerevisiae* under 5 hours of HP CO_2 at 40ºC and 4 MPa in distilled water. Efficacy decreased at lower temperatures (2 log reduction at 30ºC and 4 MPa), pressures (4 log reduction at 40ºC and 3 MPa) and shorter

times (2 logs at 1 hour at 40ºC and 4 MPa). Few bactericidal effects were observed below 20ºC at any time or temperature.

The literature on the effect of HP CO_2 on spores is contradictory. Most studies indicate a negligible effect of HP CO_2 on bacterial spores (Kamihira, Taniguchi et al. 1987; Arroyo, Sanz et al. 1997; Spilimbergo, Elvassore et al. 2002), possibly due to their low water content. In some studies, more extreme treatments have indicated a limited effect on spores. Enomoto (1997) treated spores of *Bacillus megaterium* for up to 30 hours, and found an optimum pressure for killing spores which gave a 7 log reduction. This treatment, at 6 MPa and 60ºC, was more effective at killing spores than higher or lower pressures. He theorized that at pressures <6 MPa, treatments were not vigorous enough, and higher pressures caused spores to flocculate; providing a protective effect. A 30 hour treatment is too long for most food applications, but may be suitable for pharmaceuticals.

Fungal spores appear to be more sensitive to HP CO_2 treatment than bacterial spores (Shimoda, Cocunubo-Castellanos et al. 2001). *Aspergillus niger* spores treated at 10 MPa and 52ºC, had a D value of 0.16 minutes. There was no reduction in a control treated under equivalent pressure and temperature conditions in the absence of CO_2. Efficacy was highly dependent on temperature (D of 1.74 min at 44ºC). While the results obtained from *Aspergillus niger* suggest that certain spores are sensitive to treatment, *Aspergillus* is typically not a problem organism in most food applications.

1.3.1 Mechanisms for Bactericidal Effect

A primary mechanism behind this CO_2's inhibition on bacteria is its ability to lower the interstitial pH of bacterial cells (Dixon and Kell 1989).

When CO_2 dissolves in aqueous solution, a portion reacts with water to form carbonic acid.

$$CO_2 + H_2O \leftrightarrow H_2CO_3$$

DP CO_2 is able to penetrate the cell walls of bacteria at a higher rate than in its gaseous state, and is most efficient near its critical point of 7.2 MPa and 31°C (Rizvi, Daniels et al. 1986). Supercritical fluids are better solvents and have lower surface tensions then their liquid counterparts and would likely disperse more quickly through the bacterial wall.

Bacteria can survive in an acidic environment, but the internal pH of these cells must remain near neutrality (Hong and Pyun 1999). To compensate for acidic surroundings, cells have to maintain a pH gradient between the internal and external environment. This is accomplished by actively pumping hydrogen ions from the interstitial fluid to the outside of the cell. Because of the high permeation of CO_2 through the cell membrane, the pumping system is overwhelmed and the interstitial pH is reduced. This acidification results in the destruction of the cell, probably by the denaturing of cellular enzymes (Ballestra, Dasilva et al. 1996). Dillow (1999) claims that the reduction of internal pH is likely to be the main source of inactivation, citing that pressure alone (up to120 MPa) is generally insufficient to cause the same bacterial inactivation as can be had with CO_2.

The application of HP CO_2 to bacterial cells has been shown to produce a non-linear death curve (Ballestra, Dasilva et al. 1996; Shimoda and Osajima 1998). These curves contain a short stationary phase followed by a logarithmic decrease in the number of bacteria. Increased temperatures and pressures served to shorten the stationary portion of the curve as well as

increase the rate of the death phase (Ballestra, Dasilva et al. 1996). Ballestra theorized that the stationary phase was caused by the sub-lethal stressing of cells by the accumulation of CO_2 within the cell wall. When the amount of CO_2 accumulated within the cell reached a critical value, the pH change was sufficient to denature the interstitial proteins of the cell causing death. The shortened stationary phase observed at increased temperatures and pressures was due to a faster rate of CO_2 diffusing into the cell.

Another force, which may inactivate the bacterial cell, is the rapid depressurization of the system. By this theory, the quick expelling of gas compromises the structural integrity of the bacterial cell membrane making the cells rupture at the moment of depressurization (Enomoto, Nakamura et al. 1997).

Nakamura's (1994) results with from disrupting *Saccharomyces cerevisiae* by the use of flash depressurization of CO_2 suggested that the death of microorganisms occurred mainly due to mechanical rupture while rapidly depressurizing. Most of their evidence cited electron micrographs that show the presence of ruptured cells after treatment at 5 MPa at 40°C for 4 hours followed by a sudden pressure release.

However, the idea of an inactivation caused solely by pressure rupture of cells has fallen out of favor in recent years. Enomoto, Nakamura et al. (1997) experimented on the difference of lethality with slow depressurization compared to flash depressurization at pressures of 4 MPa. Rapid depressurization had no significant difference on microbial populations when compared to slow pressure release. This would suggest that at pressures of less than 4 MPa, the death due to explosive decompression was minimal. The amount of protein released from the treated yeast cells was three times higher

than the incubated control, indicating that dissolved proteins may be the cause of cell death. Therefore, he theorized that most kill took place during pressurization, not during the pressure release.

This experiment does not completely discount the explosive decompression theory however. Pressures used were relatively low so pressure drops greater than 4 MPa may generate increased kill. Secondly, even though the depressurization was termed “explosive”, it took nearly a minute for the pressure to completely discharge, potentially negating more rapid depressurization effects.

Ballestra (1996) used electron microscopy to look at the cell walls of *E. coli* before and after HP CO_2 treatment at 5 MPa. Ballestra observed significant cell wall destruction, with more than 75% of the cells being visibly ruptured. However, even though 25% of the cells had intact cell walls, there was only 1% viability in the sample. This suggests that either the holes in the cell wall were too small to be detected by electron microscopy, or another factor was at work.

Dillow (1999) found bacterial reduction increased with the inclusion of pressurization and depressurization cycles. Although the extent of inactivation in some treatments was large, there were few visible changes from scanning electron microscope photographs of the populations, a mechanism other than rapid decompression was likely responsible for cell inactivation.

Lin et al (1991; Lin, Yang et al. 1992) pressurized yeast cells up to 34 MPa and theorized that the observed cell death was due to two previously mentioned effects working synergistically: rapid depressurization and solvent effects of HP CO_2. By measuring the amount of cellular proteins released from the cells after treatment Lin found that the HP CO_2 was able to dissolve a

portion of the cell proteins. Rizvi and Daniels (1986) explain that SC CO_2 is an excellent extraction solvent, and more soluble in the non-polar compounds. This property of CO_2 makes it suited for the extraction of compounds from a cell membrane. Lin (1991) suggested that this solvent mechanism was responsible for cell but was most effective at the moment of depressurization. In this way, the expansion of CO_2 at pressure release was able to rapidly transfer the extracted intercellular proteins out of the bacteria. To take advantage of cell death due to depressurizing, Lin (1992) used several pressure release and repressurization steps to maximize efficacy. A significant increase in protein release and rate of bacterial inactivation was observed using repeated cycling.

The amount of dissolved CO_2 in the system has also been shown to be an important factor in the effectiveness of the treatment (Shimoda and Osajima 1998). (Tomasula, Boswell et al. 1999) found that the solubility of CO_2 in milk increased with higher pressure and lower temperatures, analogous to solubility in water (Weibe 1940). Although the solubility data between water and milk was similar, in absolute terms, CO_2 solubility in milk was marginally lower than that of water over the range of pressure/temperature combinations studied (25-50 ºC, up to 7 MPa) (Tomasula, and Boswell et al. 1999).

Shimoda and Yamamoto (Shimoda, Yamamoto et al. 1998) found a continuous flow process in which CO_2 and product were continuously mixed and pressurized was more effective at killing bacteria than a batch process. *Lactobacillus brevis* was completely inactivated with HP CO_2 at 0.16 g/cm^3 in a continuous reactor and 0.9 g/cm^3 in a batch reactor. The degree of cell death was correlated to the amount of dissolved CO_2 in both the batch and

continuous reactors. The increased kill in the continuous treatment was attributed to two factors. First, the continuous system dispersed CO_2 more rapidly than the batch system, leading to a higher concentration of dissolved CO_2. Secondly, the continuous processes contained a more explosive decompression than the batch system, a force that has been mentioned as a mechanism for inactivation.

1.4 Effect on Enzymatic Activity

Aside from the destruction of bacteria, thermal processing of food also serves to inactivate enzymes. Enzymes in food are responsible for reducing quality in a variety of ways. The breakdown of cloud in juices, the browning of cut produce, and the hydrolysis of fats and proteins are a few of the major negative effects enzymes have on foods. However, when compared to the numerous papers on CO_2's bactericidal effect, little work has been done on the effects of HP CO_2 on enzymatic activity.

The limited work that has been done on enzyme inactivation suggests HP CO_2 processing is potentially able to reduce the active enzymes in a food. Addition of HP CO_2 causes at least three stresses that could potentially change the enzymatic activity of the sample.

Pressures around 310 MPa can cause irreversible damages to the secondary structure of proteins at room temperature. Pressures below this level generally produce no change or changes to proteins that are reversible upon depressurization (Hendrickx, Ludikhuyze et al. 1998). Balney et al. (Balny and Masson 1993) demonstrated that lower pressure denaturation, around 207 MPa may be reversible. This reversibility is affected by external sources such as temperature, pH, and presence of sugars, salts, or other

additives. Further investigation showed that the mechanism behind inactivation comes from the break down of the tertiary structure of proteins, which begins at 207 MPa (Balny and Masson 1993).

Most enzymes are inactivated by pressure without CO_2 at room temperature in the 207 – 608 MPa range (Knorr, Hendrickx et al. 2002). The pressures required for enzyme inactivation are considerably higher than the pressures typically used in HP CO_2 processing. Therefore, pressure alone would not be expected to account for the inactivation of enzymes at the pressures normally used in HP CO_2 processing.

All enzymes have a pH optimum due to the degree of ionization of the amino acid side groups at its active site, changing the ability to accept a substrate (Schwimmer 1981) Enzymes may also be inactivated as pH decreases, but stability and optimum are not related (Schwimmer 1981). Therefore, the change in pH caused by the addition of HP CO_2 would have an effect on both the kinetics and the stability of an enzyme. Individual enzymes exhibit a range of pH stabilities from a pH of below 2 to above 13 (Fennema 1996). Because enzymes possess a range of acid stabilities and pH optima, the effect that acidification has on reaction rate is difficult to predict.

The final way in which high pressure CO_2 processing may change the enzymatic activity of a food is by a change in substrate structure. Substrates require a suitable conformation for reaction with an enzyme. An alteration of the substrate's conformation could inhibit the rate of the enzyme. A conformational effect also would include an alteration of the microstructure of a substrate. A gross change in the way a substrate is oriented may increase or decrease its availability to the enzyme. For example, the pressures generated in this process are capable of producing high shear, which can

fracture the microstructure of a substrate. This break up would increase the substrate's surface area making it more available to the target enzyme, and increasing the rate of enzymatic activity.

The use of HP CO_2 to inactivate enzymatic activity has been met with mixed effectiveness. Kamihira (1987) treated an enzyme preparation of lipase and α-amylase with CO_2 at 21 MPa and 35°C for 2 hours. After this treatment, lipase and α-amylase activities had 88% and 121% of original activity respectively.

Taniguchi et al (1987) applied CO_2 at 20 MPa and 35ºC for one hour to a non-aqueous mixture of enzymes as well as a mixture suspended in a 3% ethanol solution. Taniguchi's data demonstrate little effect of HP CO_2 in treatments without water across enzyme types. In addition, the presence of other substances such as ethanol may increase the effectiveness of HP CO_2 treatment.

Table 2. Residual enzyme activity after SC CO_2 treatment at 20 MPa and 35°C for 1 hour. Results are shown with and without a 3% ethanol solution (Taniguchi et al 1987)

Enzyme	**CO_2**	**CO_2 + 3% Ethanol**
amylase	94	96
glucoamylase	102	96
B-Galactosidase	98	98
Glucose Oxidase	97	93
Glucose Isomerase	102	95
Lipase	96	88
Thermolysin	101	96
Alcohol Dehydrogenase	97	87
Catylase	90	96

Aereola (1991) produced an 84% reduction in pectinmethlyesterase (PME) activity in orange juice after 2 hours, and 100% reduction after 4 hours of treatment at 45ºC and 30 MPa. Balaban et al (1991) corroborated this research by showing CO_2 treatment of orange juice resulted in more inactivation of PME than CO_2 treatments at atmospheric pressures. From these results, Balaban concluded that CO_2 treatment allows inactivation of enzymatic activity at temperatures where thermal treatment was insufficient.

Corwin and Shellhammer (2002) investigated the effect of various CO_2 treatments on PME and polyphenol oxidase (PPO). This experiment's design differed from most of the literature due to the high pressures and short times (3 minutes) used. Both enzymes were subjected to a treatment of 0, 500 and 800 MPa at 25ºC or 50ºC with or without CO_2. The enzymes reacted differently to the effect of HP CO_2. PME was relatively resilient resistant to the effects of CO_2 and only one treatment had significant inactivation over a control (500 MPa, at 25ºC). However, PPO treatments containing CO_2 showed significant inactivation over the controls treated with pressure alone at all pressures and temperatures. PPO and PME therefore have markedly different resistances to the effect of CO_2, with PPO being more labile.

Park (2002) looked at the effects on enzyme activity of carrot juice after 5 minutes of HP CO_2 treatment (up to 4.9 MPa) followed by 5 minutes of high pressure treatment (up to 600 MPa) without CO_2. He found that all three enzymes tested (Lipoxygenase (LOX) > PPO >PME) showed decreased activity after treatment. HP CO_2 treatment alone dropped enzyme activity, but the effect increased upon the addition of high pressure. Five minutes of HP CO_2 at 4.9 MPa decreased LOX to 16%, PPO to 32%, and PME to 37% of the

original activity. After 600 MPa of pressure treatment LOX, PPO, and PME activity dropped to 9%, 12%, and 25% respectively.

After SC CO_2 treatment at 25 MPa at 35°C for 30 minutes, Ishkawa (1996) recorded the change in the residual α-helix structure of several enzymes. The residual α-helix contents of lipase, alkaline protease, acid protease, and glucoamylase were 62.9, 31.3, 37.6, and 12.4%, respectively. This structural change was highly correlated with the residual enzyme activity. Thermally treated enzymes had an increased reversion rate of α-helix structure when compared to CO_2 treated samples, suggesting different inactivation mechanisms.

Tejdo (2000) tested the protective effect of adding sucrose or buffer to an enzyme preparation. Under all treatment conditions between the two enzymes tested, peroxidase (POD) was more resistant than LOX. For both enzymes, each 10% increase in sucrose concentration raised enzyme activity approximately 15% after treatment. Increasing the buffer capacity of the enzyme preparations (pH range 4-9) led to significantly higher enzyme activities than unbuffered controls. These findings suggest that a complex food system with solutes and buffers would have a protective effect on endogenous enzyme activity when treated with CO_2.

1.5 Effect of Carbon Dioxide on Dairy

CO_2 has been reported to occur naturally in milk at levels of approximately 90 ppm (Vandenberg 1979). The bacteriostatic effects of higher concentrations of CO_2 in dairy have been known for some time (King and Nagel 1967). The addition of CO_2 to dairy products as a preservation technique has been used in such products as cottage cheese (Chen and

Hotchkiss 1991). This technology's effectiveness is best appreciated when its rapid adoption and widespread use in the dairy industry is taken into consideration.

1.5.1 Endogenous Dairy Enzymes

Endogenous milk enzymes play an important role in the shelf life of milk and dairy products. The two broad enzyme types responsible for a majority of the degradation of milk quality are lipases and proteases (Ma, Ryan et al. 2000).

The principal lipase of concern, lipoprotein lipase (LPL), is associated with the casein micelles in the skim fraction of the milk (Fox 1992). Mechanistically, lipases produce free fatty acids by cleaving the ester bond connecting a fatty acid to a glycerol molecule in a triglyceride. Free fatty acids have a pungent aroma, which results in a "rancid" note in the milk. While this may be considered an off-flavor in fluid milk, many dairy products (such as blue cheese) benefit from a characteristic "rancid" flavor.

Traditional pasteurization preserves milk not only because of the reduction in the number of spoilage microbes, but also because of its effective inactivation of LPL. LPL is one of the more heat labile enzymes, and is inactivated at 60ºC (Andrews, Anderson et al. 1987).

The next major category of endogenous milk enzymes are the proteases. Of principle importance is the protease plasmin (Sousa, Ardo et al. 2001). Plasmin's precursor, plasminogen is changed to plasmin by specific enzymes responsible for the conversion. The activation of plasmin is kept in

check by other enzymes that inhibit the plasminogen activators (Fox 1992). Standard high temperature short time processing of milk (72°C, 16 seconds) destroys the inhibitors of the plasminogen activators, but not plasmin itself. By inactivating inhibitors, traditional thermal pasteurization increases the conversion of plasminogen into plasmin, effectively increasing the rate at which proteolysis occurs. Plasmin is extremely heat stable (Kaminoga.S, Mizobuch.H et al. 1972). It would take very high temperatures to inactivate it; activity even remains in ultra high temperature pasteurized milk (282°F, <2 seconds). Processing milk at temperatures above this level cause severe organoleptic defects (Iwatsuki, Mizota et al. 2000), so plasmin activity remains a problem even in thermally treated milks.

1.5.2 Effect of Carbon Dioxide on Dairy Enzymes

No work has yet been done on the stability of dairy enzymes after HP CO_2 treatment. As stated previously, HP CO_2 processing may change the pressure, pH, and substrate of a system. One may predict the effect of HP CO_2 treatment by looking at the effect each of these three stresses has on milk individually.

Scollard, Beresford et al. (2000) showed that plasmin is pressure stable to 621 MPa. Addition of denatured β-lactoglobulin destabilizes the enzyme, but this only reduces the pressure necessary to inactivate the plasmin to 400 MPa. Argyriou (1998) showed a 45% reduction in lipase activity at 200 MPa, but even at 800 MPa, 20% activity remained. If pressure alone were used to treat milk, extremely high pressures would be needed to inactivate endogenous enzymes. However, most HP CO_2 treatments rarely attain pressures adequate for this to happen.

Because of the high pressures involved, determining the pH in the apparatus being tested proved impossible. In previous studies, the high pressure CO_2 processing of milk has been calculated to lower the pH of milk rapidly over the first 1.5 MPa, and quickly comes to an asymptote at a pH of approximately 4.8 (Hofland, van Es et al. 1999). The inability of milk to attain lower pH values, even at higher pressures, is due to of its high buffering capacity. Another observation of Hoffland's is that this ultimate pH decreases with decreasing temperature as a result of increased CO_2 solubility. This ultimate pH does not drop below the isoelectric point of casein (pH 4.6) up to pressures of 6 MPa (even though Hoffland observed casein precipitation at these treatments).

Plasmin is stable to pH 4.0 (Kaminoga.S, Mizobuch.H et al. 1972). Additionally, the process of making many dairy products drops the pH of milk below 4.6 and proteolysis and lipolysis still occur. Therefore, it is unlikely that pH alone will inhibit the primary proteolysis and lipolysis to a great extent.

If processing altered the substrate of an enzyme, the rate of reaction may change even though the enzyme may be viable. As pH in milk decreases to the isoelectric point, the previously negative surface charges of the casein micelle surface are neutralized. The disappearance of the electrostatic repulsions that previously held caseins apart causes the micelles to clump together to form a precipitate. Plasmin may not be able to act upon a precipitate at the same rate as the micellular protein and may change the apparent activity of plasmin.

Homogenization of fat globules changes their structure significantly. Globules inherently exist in a range of sizes (about 0.1 to 10 μm), and most of the volume of the milkfat lies in the globules above 1 μm.

The majority of the triacylglycerol content of milk occurs in fat globules 1-10 μm in diameter. In their native state, these large globules reduce the surface area of the fat, protecting it from the action of LPL. Homogenization is typically a two-step process that takes place at 13.8 and 3.4 MPa. The process breaks up the fat globules, increasing the surface area available to enzymatic action and exponentially increases the rate at which enzymatic lipolysis proceeds. Unless some treatment is able to destroy the activity of the lipase after homogenization, lipolysis will proceed at a rapid rate.

1.5.3 Precipitation of Casein

Although HP CO_2 processing has the ability to kill bacteria while inactivating enzymes (Hong and Pyun 2001), and CO_2 has effectively been used in the dairy industry, HP CO_2 remains unused in commercial applications. One hurdle to the incorporation of HP CO_2 into the dairy industry rises from the effects it may have on casein proteins.

By definition, a casein protein in milk is one that is precipitated out at the isoelectric point of pH 4.6 (Fox 1992) When CO_2 dissolves in aqueous solution, a portion reacts with water to form carbonic acid.

$$CO_2 + H_2O \leftrightarrow H_2CO_3$$

If enough CO_2 is incorporated into the milk to reduce the pH to 4.6, the casein in the samples will precipitate out. Processes which utilize CO_2 for precipitation of casein have been developed for industrial manufacturing (Tomasula 1997). For most other dairy applications, the precipitation of casein would be a negative consequence of processing.

If HP CO_2 treated milk is to be used for fluid milk consumption it would have to avoid precipitation, as particles of precipitated casein are likely to be poorly received by consumers. Cheeses made from CO_2 treated milk have been shown to have improved renneting characteristics and slightly higher cheese yields (Ruas-Madiedo, Alonso et al. 2002). However, curd produced from milk with partially precipitated protein would not be satisfactorily firm. Subsequent cheese making would experience free fat loss and excessive fines, contributing to low yields.

Precipitated casein has applications both as a food ingredient and as an industrial product. Typically in this industrial process, casein is precipitated in milk by the addition of a mineral acid such as HCl. The curd is then separated from the whey and dried. While this method is effective, casein precipitation by CO_2 would have several advantages. First, CO_2 used as the acidifying agent can be removed (and potentially recovered) from the whey after processing adding value to the uncontaminated sweet whey. Additionally, no acid remains to contaminate the curd, leaving a purer casein product.

Tomasula and Craig (1995) developed a batch system where skim milk was exposed to high levels of CO_2. In this system, pressures and temperatures between 2.8 and 5.5 MPa 38ºC and 49ºC were tested. Shorter holding times resulted in the same yield as longer ones. This suggested that residence time is not a significant factor in casein precipitation, and once the isoelectric point is reached, all susceptible casein is precipitated. While the effect of time was not significant, temperature of treatment was an important variable in casein production. The optimal coagulation temperature recommended for casein manufacture was found to be 38ºC. Temperatures

below this level resulted in a wet, friable solid that dissolved soon after treatment.

Whey proteins are more resistant to the effects of HP CO_2 than casein proteins. Experiments on skim milk found that at all processing parameters investigated, whey proteins were not precipitated (Calvo and Balcones 2001). Caseins were readily precipitated under the same conditions. The mildest treatment (2 MPa, 30 min, and 40ºC) precipitated 35% of the caseins, while the most severe treatment (5 MPa, 180 min, 40ºC) precipitated 87% by weight. The experimental apparatus used by Calvo differs from the one used in the present work in that his system was batch rather than continuous.

Jordan and Lay (1987) studied the ability of CO_2 to precipitate casein from skim milk by the addition of CO_2. At 3.5 MPa and 50ºC the researchers were able to precipitate 99% of the casein in skim milk. When a range of pressures were used, Jordan found the pressures required for precipitation decreased as the temperature increased. Although the lowest temperature investigated was 40° the trend of the data imply that processing at lower temperatures may avoid precipitation. This paper also suggests that time is not an important factor of casein precipitation. Because of the batch system design, there was a long come up time of 7-10 minutes. Yields obtained from runs that were depressurized immediately after attaining operating pressure were the same as those that included a holding period. Identical yields with and without a holding time echoes the findings of Tomasula (1995).

The physical nature of the curd produced changed with the pressure used. Higher pressures resulted in a firm, rubbery curd while more mild treatments produced a curd that was softer and more easily friable.

1.7 Effect of CO_2 on Dairy Vitamin Stability

Typically, fluid milk sold in the United States is fortified with vitamins A and D prior to pasteurization. While this process is not mandatory according to the Pasteurized Milk Ordinance (FDA 1999), it is performed to a large percentage of fluid milk. Therefore, the stability of these fortifications in milk having undergone this process should be addressed. No research has been done on the stability of vitamins in milk after HP CO_2 processing, but an assessment can be made after looking individually at the basic effects of the DP CO_2 system.

A few studies have looked at the impact of CO_2 at atmospheric pressure on the stability of different vitamins. Ruas Madiedo et al (1996) acidified milk with CO_2 to pH 6.2 and investigated residual vitamin stability. Fat-soluble vitamins retinol, β-carotene and α-tocopherol were measured for 8 consecutive days from 4 days before to 4 days after standard HTST thermal pasteurization. The CO_2 treated sample when compared to a HTST control had significantly better preservation of retinol and β-carotene . Researchers measured a 2% rise in α-tocopherol content for the CO_2 treated sample compared to a 6% decrease in the non-treated sample. Sierra et al (1996) also failed to find a statistical difference between vitamin activity in control and CO_2 acidified milk samples.

Though pressures in the present work do not reach levels of true high pressure processing (upwards of 100 MPa vs. 62 MPa for our system) pressure will not likely change vitamin stability. (Garcia, Butz et al. 2001) showed no difference in the vitamin activity of fat-soluble vitamins through the use of high pressure processing at room temperature and up to 400 MPa. When working with fresh orange juice, de Ancos (2002) was able to show

high-pressure treatments at 350 MPa produced significant increases of 20-43% in the carotenoid content (from 3.99 to 4.78-5.70 mg /l)

None of these forces taken alone has potential to decrease the vitamin content of milk. Therefore, it is likely that unless some great synergistic effects between carbonation, pH, and pressure exist, the vitamin content of DP CO_2 processed milk should remain intact.

CHAPTER TWO

GOAL AND OBJECTIVES

2.1 Goal

The goal of this work was to characterize chemical changes that occur in milk after continuous DP CO_2 processing.

2.2 Objectives

1 Determine the affects of CO_2 pressure/temperature interactions on the milk lipases and proteases after exposure to high pressure CO_2 between 7 and 62 MPa and 15° to 40°C

2 Determine if a continuous addition of CO_2 has a different effect on this chemistry than processing raw milk that has been pre-carbonated.

3 Determine what effect the high pressure CO_2 process has on the particle size distribution of the treated milk.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

3.1.1 Milk

Raw Holstein milk was delivered within 24 hours of milking from the Cornell Teaching and Research Center by bulk tanker truck. Raw whole milk was used untreated, and raw skim was obtained by cold separation at the Cornell University Dairy (Ithaca, NY Plant #36-8832).

3.1.2 Carbon Dioxide

Carbon Dioxide (CO_2) for the continuous process was acquired from Praxair (Chicago, IL) in standard high-pressure cylinders equipped with eutectoid tubes. The tubes allowed for the delivery of liquid CO_2 from the bottom of the tank. Carbon dioxide used in the pre-carbonation experiments (Airgas Midatlantic, Elmira, NY) was also in shipped high-pressure cylinders, but had no eutectoid tubes.

3.2 Processing Description

3.2.1 Pre-carbonation

In some experiments, milk was pre-carbonated before processing using at 2°C using a continuous flow inline CO_2 injection system. This system was modified from a countercurrent stainless steel tubular heat exchanger (internal diameter = 0.5 cm) cooled by circulating ice water. Skim milk was pumped through the system with a peristaltic pump and CO_2 was injected through a

stainless steel tube (internal diameter = 0.8 cm) inserted through a tee-fitting perpendicular to the milk flow immediately after the feed pump. The residence time of the milk was approximately 20 seconds. Four carbonation levels between 0 and 2400 ppm were obtained by adjusting the flow rate of the CO_2 while maintaining a constant 900 ml/min flow rate. After carbonation, milks were stored in stainless steel cans overnight at 4°C to allow for equilibrization. Previous tests indicated no loss of CO_2 during this storage time.

3.2.2 Dense Phase Processing

Milk processing was performed on proprietary equipment developed by Praxair Inc (Chicago, IL) capable of continuously treating milk with liquid CO_2 at pressures up to 69 MPa. The machinery was housed in the Food Processing and Development Laboratory at Cornell University. Most simplistically, this system pumped CO_2 and milk up to a set pressure, held it in a holding tube, and released the pressure back to atmospheric. Processed samples were collected at the end.

Raw milk pumped from the feed tank, combined with a stream of CO_2 at a tee-junction approximately 0.5m from the diaphragm pump (figure 1). From there, the milk/CO_2 blend flowed through a static mixer to assure that the CO_2 was sufficiently mixed with the milk.

The mixture was raised to operating pressure by a reciprocating intensifier pump (Haskel MLP-25/46D) that pressurized the milk/CO_2 mixture to 202 times the supplied air pressure. The operating pressure was controlled by adjusting a back pressure regulator (BPR1) at the end of the 12.2m holding tube. This tube, which immediately followed the intensifier pump, was made with 0.787cm ID stainless steel tubing (total volume 594ml) and surrounded by

heating tape. Temperature in the holding tube was monitored by three thermocouples at the beginning, middle, and end. The middle thermocouple provided the feedback for a temperature controller (Love Controls Series 16A., Michigan City, Indiana) to adjust the temperature of processing.

After the mixture passed through BPR1, it entered the first of two pressure reduction sections. These sections were constructed of the same tubing and fitted with a temperature controller and heating tape. The heating tape prevented milk from freezing by adiabatic cooling lowering the temperature of the milk below 0°C. Pressure on each section was adjusted with a backpressure regulator at the end of each 1.5m tube. The regulator valves were set to 3.79 MPa (BPR2) and 1.72 MPa (BPR3) for all experimental runs.

Upon exiting the final pressure reduction section, the mixture flowed through a 0.75m section of 0.635cm ID tubing (Tygon model 106 Akron, OH) into a large separator tank, where undissolved CO_2 flashed off the product.

3.3 Experimental Procedure

A typical inline carbonation run included an initial sanitization step, product processing, and a final cleaning and sanitization as follows:

Initial Sanitizing

1. Turn on electrical disconnect and instrument power switch.
2. Turn on chiller set at 0 °C.
3. Make sure Prime Valve HV-2, Air Inlet Valves HV-5 & HV-3, and CO_2 cylinder valve are closed.
4. Open BPR-1, 2, and 3 fully.

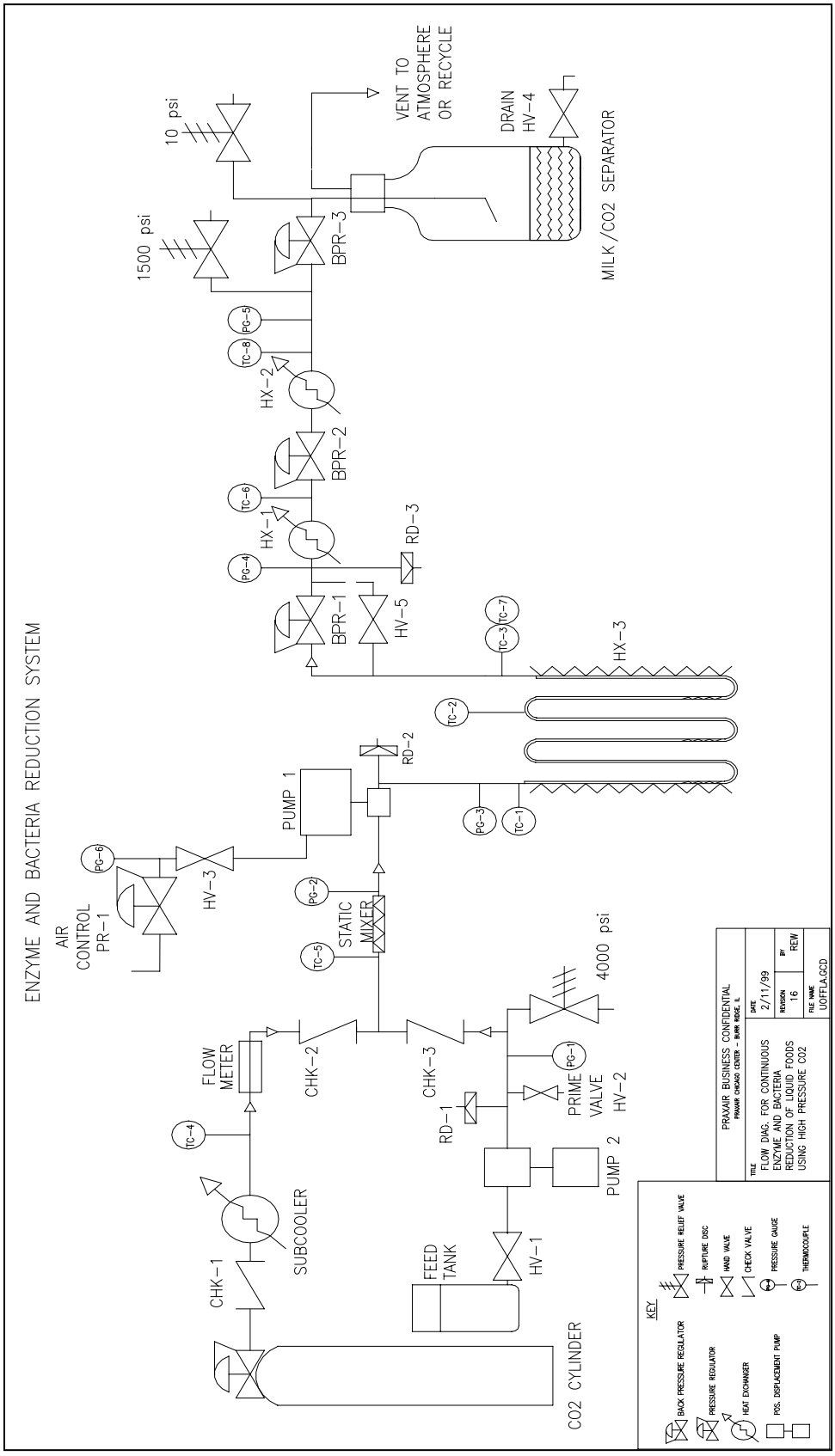

Figure 2 Diagram of the processing system courtesy of Praxair

5. Connect the Product Feed Tank, filled with water, to the Product Supply Pump (Pump 2). Open Product Feed Tank outlet valve HV-1.
6. Set stroke-length on Pump-2 to 84%
7. Connect Product/CO_2 Separator. Close drain Valve HV-4
8. Open Air Inlet Valve HV-5. Adjust PR-1 regulator to a pressure, which will allow the pump to reach the desired operating pressure. (The ratio of fluid pressure to air pressure is 202:1 on this pump. Take the desired process pressure and divide by 202. Set air pressure to this number.)
9. Start the Product Supply Pump (Pump-2) from the electrical cabinet.
10. Open Prime Valve HV-2 until water appears in steady flow. Drain into a suitable container.
11. Close HV-2.
12. Temporarily turn off product feed pump, connect feed line to sanitizing solution (28.6 g Tri-Chloro-Ocide (Ecolab, St. Paul, Minnesota) / 3.8L H_2O)
13. Open HV-2 and allow solution to drain into a suitable container for 30 seconds of contact time
14. Close HV-2 and allow solution to run through the unit for 20 minutes

Milk Processing

15. Turn off supply pump and connect supply tube to water flush
16. If parameters tested are below ambient, ice water was used as the product feed, and was allowed to flow through the machine for 30 minutes.
17. Start temperature controllers for Stage 1 and Stage 2 Pressure Reduction by pressing Auto/Man button on each for three seconds. Set them to desired level.
18. Open Air Inlet Valve HV-5, and adjust HV-3 on Pump-1 until cycling of the pump begins.
19. Adjust BPR-3 until PG-5 reaches 1.72 MPa.
20. Adjust BPR-2 until PG-4 reaches 3.79 MPa.
21. Adjust BPR-1 and HV-3 until PG-3 reaches desired process pressure. Pump 1 should be running at approximately 70 cycles per minute.
22. With the cylinder regulator closed, open the CO_2 cylinder valve. Set the regulator to 4 MPa outlet pressure.
23. Adjust air control valve HV-3 and the regulator on the cylinder to set CO_2 flow rate (fluctuating above 15g/min)
24. Turn off feed pump, switch feed line to the milk, restart feed pump
25. Allow at least 10 minutes of steady state flow before sample collection
26. Collect sample at desired parameter
27. Repeat previous two steps for each processing parameter tested

Post Treatment Cleaning

28. Turn off feed Pump, connect tube to rinse water, adjust pump to 100% flow, restart pump
29. Adjust all temperature controllers to 40°C
30. Open HV-2 for 30 seconds of rinse contact time, close valve
31. Flush system for 30 minutes
32. Turn of feed pump, switch feed tube to Conquest™ cleaning solution (36 ml Conquest™ (Ecolab, St. Paul, Minnesota) to 3.8 L of water) pH 13, restart pump
33. Open HV-2 for 30 seconds of rinse contact time, close valve
34. Run cleaning solution through system for 30 minutes
35. Turn of feed pump, switch feed tube to rinse water,
36. Open HV-2 for 30 seconds of rinse contact time, close valve
37. Run rinse water through system for 30 minutes
38. Turn of feed pump, switch feed tube to Monarch CIP solution (95 ml Monarch Acid CIP (Ecolab, St. Paul, Minnesota) to 3.8 L of water), restart pump
39. Open HV-2 for 30 seconds of rinse contact time, close valve
40. Turn of feed pump, switch feed tube to rinse water,
41. Open HV-2 for 30 seconds of rinse contact time, close valve
42. Run rinse water through system for 30 minutes

43. Turn of feed pump, switch feed tube to sanitizing solution (28.6 g Tri-Chloro-Ocide in 3.8L H_2O), restart pump
44. Open HV-2 for 30 seconds of sanitizing contact time, close valve
45. Run sanitizing solution through system for 30 minutes
46. Turn of feed pump, switch feed tube to rinse water,
47. Open HV-2 for 30 seconds of rinse contact time, close valve
48. Run rinse water through system for 30 minutes

When pre-carbonated milk was used, steps 23 and 24 were omitted, and the remainder of the operation was identical to the protocol above.

Immediately after processing, all samples were degassed in a rotary evaporator for 15 minutes in a 40°C bath at 85 kPa vacuum. Each treatment then had 0.02% potassium dichromate added to as a preservative, was mixed thoroughly, and divided into 40ml snap-top disposable vials (Capitol Vial., Fultonville, NY) common in the dairy industry. The 0.02% potassium dichromate was used to prohibit bacterial growth without inactivating enzymatic activity (Ngjwaihang 1982).

Samples were incubated in a 25° incubator (Precision Scientific., Winchester, VA) free of direct light for up to 3 days.

3.4 Analytical Methods

3.4.1 CO_2 Content

CO_2 content was determined according to methods described in Martin and Hotchkiss (2002). A 25g milk sample was transferred to a 100 ml amber

septum bottle with a 3.5 cm stir bar, sealed with a septum, and allowed to equilibrate to room temperature. With a syringe, 10 ml of pH adjusted, degassed distilled water was added to the bottle. The bottle was then injected with 5 ml of degassed 1N sulfuric acid and stirred for 5 minutes. A 10 cc sample of headspace was drawn from the bottle with an air-tight Luerloq fitted syringe and injected into an infrared CO_2 analyzer (Mocon, Pac Check 200, Minneapolis, MN) and concentration of CO_2 determined by comparison to a previously prepared standard curve.

3.4.2 Particle Size Analysis

Particle size distribution was determined using a Malvern Mastersizer E (Malvern Instruments., Worcestershire, UK). Results were recorded by the proprietary software (Masterzizer ver 1.2., Worcestershire, UK). Samples were added to 40°C water in the beam tank until an obscuration of 19-25% was obtained, at which point the measurement was read.

3.4.3 Proteolysis Assay

Proteolytic enzymes cause a decrease in casein as a percentage of total nitrogen (CN%TN) over time (Ma Ryan 2000, Santos 2001) so CN%TN was taken as a measure of proteolysis activity. Casein nitrogen was calculated by the difference in Kjeldahl determined total nitrogen (TN) (AOAC method number 991.20) and Kjeldahl determined non-casein nitrogen (NCN) content (Lynch and Barbano 1999).

$$CN\%TN = \frac{(TN - NCN)}{TN}$$

TN was measured at time 0 for each processing treatment and used as the TN value for all CN%TN calculations for that treatment. NCN samples were prepared by dividing treated, dichromate preserved, degassed milk into 40ml snap-top vials. After storage at 0, 24, 48, and 72 hours in a 25°C incubator, samples were removed and prepared for Kjeldahl analysis. All samples were run in duplicate.

3.4.4 Lipolysis Assay

The degree of lipolysis, taken as an index of lipolytic activity, was assayed by measuring the amount of free fatty acid (FFA) in a milk sample over 4 days of storage at 25°C. Processed, preserved, degassed milk was divided into 40ml snap-top vials. Samples were stored for 0, 24, 48, and 72 hours at 25°C. Upon removal from a 25°C incubator (Precision Scientific, Winchester, VA), samples were immediately placed in a -40° blast freezer, and kept there until the day of analysis. Duplicate measurements were taken, and all samples from a single experiment were run on one day using the same palmitic acid standard curve.

The FFA content (expressed as acid degree value (ADV), meq FFA/kg milk) was determined by the copper soap method, (Shipe, Senyk et al. 1980) as modified (Ma, Ryan et al. 2000). In this colorimetric method, 1ml of weighed milk sample was added to a 50ml Teflon coated tube (item 05-562-16B., Fisher Scientific Pittsburgh, PA). Next, 0.2 ml 0.7N HCl was added in order to halt enzymatic activity. To each acidified tube, 4ml of copper soap reagent, and 0.2 ml of 1% Triton-X solution was added. Chloroform, heptane, methanol solvent (49:49:2) was then added to the tubes to extract the FFA. The tubes were then shaken for 30 minutes to extract the non-polar fatty acids

and to react the copper soap with the FFA. After agitation, 3.6ml of the solvent layer was added to 0.2ml color reagent and read on a spectrophotometer (Thermo Spectronic, Genesys Spectronic 20, Madison, WI) at 440nm. The reading was then compared to a standard curve produced from palmitic acid standards of known concentrations. Readings higher than the standard curve were diluted with known volumes of extraction solvent, reread, and corrected.

3.5 Data Analysis

The lipolysis and CN%TN data were entered into the SPSS statistical package (SPSS Inc. Chicago, IL; Version 11.0.0), and the General Linear Model (GLM) was performed on the data. Parameter estimates (95% CI) of the rate of lipolysis were determined from a full factorial model utilizing factors of Day, Process, and Day*Process. Duplicate measurements were made on all proteolysis and CN%TN experiments. Most experiments were repeated in duplicate except pre-carbonated experimental runs, which were run in triplicate.

CHAPTER FOUR
RESULTS AND DISCUSSION

4.1 Sample Degassing

All treated samples were degassed to remove residual carbon dioxide (CO_2). Enzymes have pH optima (Fennema 1996), so the degassing procedure removed this effect of pH on enzyme activity. Therefore, any change in enzyme kinetics was attributed to the effect of DP CO_2 processing and not residual CO_2 in the milk.

Lipolytic enzyme activity, as measured by the increase in FFA, was reduced when samples were degassed for 15 minutes at a vacuum of 85 kPa at elevated temperatures (figure 2).

Degassing at 50° and 55°C resulted in significantly lower rates of lipolysis compared to degassing at lower temperatures (figure 2). This coincides with the heat labile nature of lipoprotein lipase (Andrews 1987), the primary lipolytic enzyme in milk. Lipolytic activity after degassing at 40° and 45° was not significantly different from an undegassed control.

Regardless of DP CO_2 treatment, the pH of milk samples dropped to approximately 5.9 from a starting value of 6.67. After degassing for 15 minutes at 40°C at a vacuum of 85 kPa, all samples reached residual CO_2 concentration (figure 3) and the pH (figure 4) of the control. Thus, all CO_2 treated milk samples were degassed for 15 minutes at 40°C at a vacuum of 85 kPa. This treatment was able to remove CO_2, and raise pH to what was present in the control milk sample while maintaining lipolytic activity.

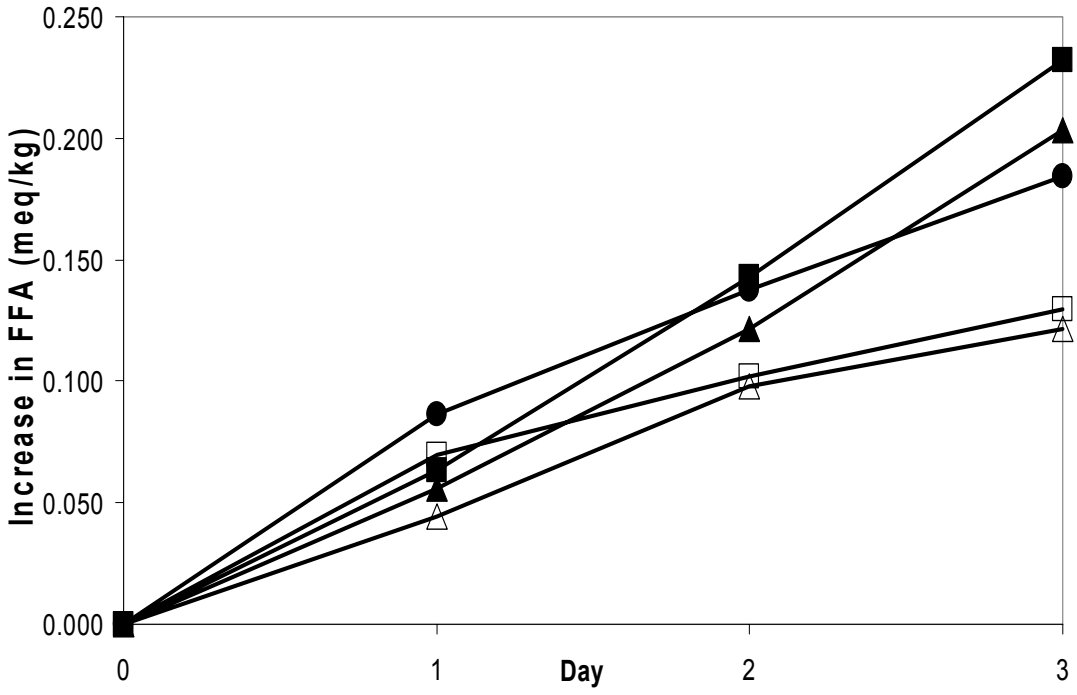

Figure 3

Mean (n=2) increase in FFA content of raw whole milk after degassing for 15 minutes at 85 kPa vacuum and 40°(—■—), 45°(—▲—), 50°(—□—), and 55°C (—△—) compared to an undegassed control (—●—)

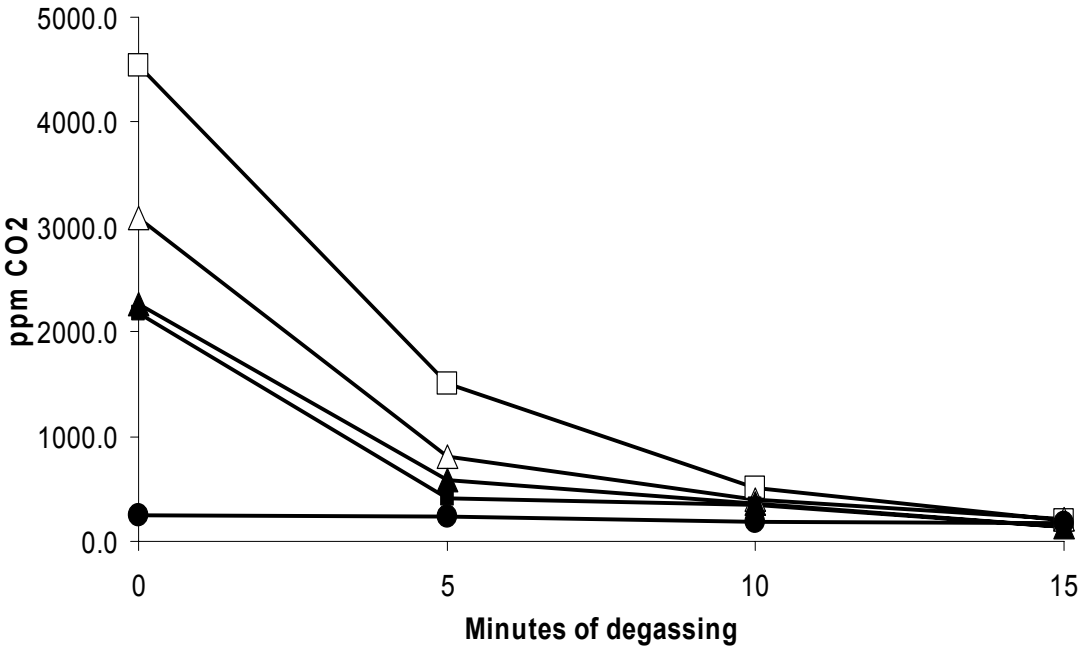

Figure 4

Mean (n=2) CO_2 content of milk after in-line treatment at 7 MPa 15° C (—□—), 7 MPa 40° C (—△—), 62 MPa 15° C (—■—), 62 MPa 40° C (—▲—), and an untreated control (—●—) after 15 minutes of degassing at 85 kPa vacuum.

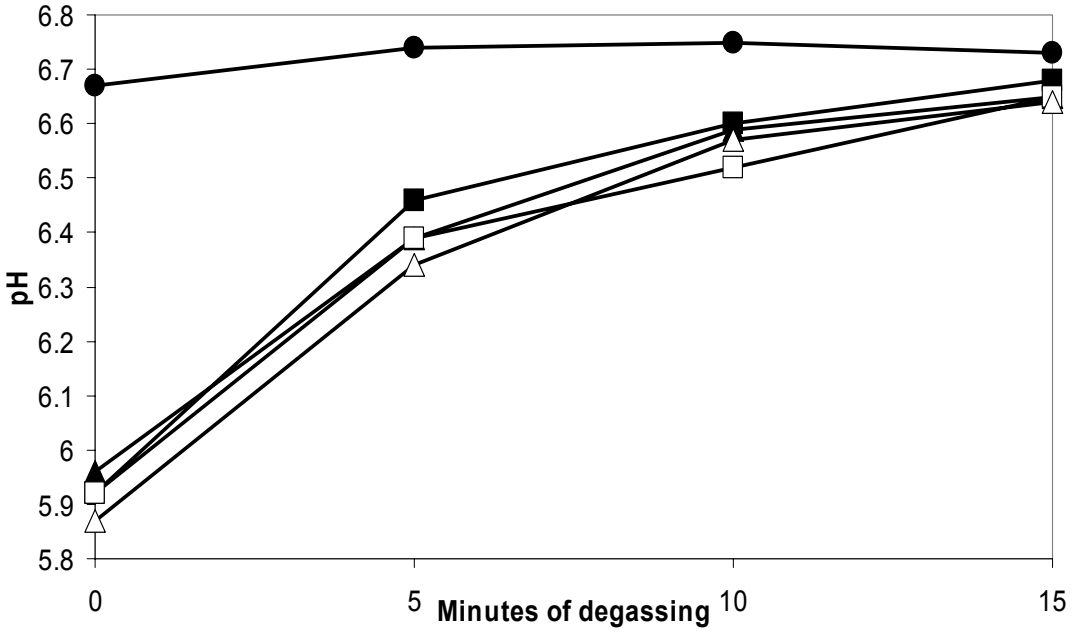

Figure 5

Mean (n=2) pH of whole milk after in-line treatment at 7 MPa 15° C (—□—), 7 MPa 40° C (—△—), 62 MPa 15° C (—■—), 62 MPa 40° C (—▲—), and an untreated control (—●—) after 15 minutes of degassing.

4.2 Dense Phase Experiments

4.2.1 Lipolysis

Application of DP CO_2 had a significant effect on the rate of lipolysis in raw whole milk (figure 5). For all experiments, the flow rates of CO_2 were 15 g/min and milk 140 g/min (0.101 CO_2/milk ratio). The FFA content of each sample was measured for the three independent treatments as well as an untreated control after 0, 24, 48, and 72 hour intervals of incubation at 25°C.

The effect of DP CO_2 processing on FFA release was temperature dependent. Treatment at 62 MPa and 15°C had essentially the same lipolytic activity as 7 MPa and 40°C, with both treatments increased the FFA content of milk to approximately 5.2 meq/kg after 3 days of 25°C incubation.

These two treatments were significantly higher than milk treated at 62 MPa at 40°C, which increased to 1.5 meq/kg. All conditions were significantly higher than the control, which increased in FFA content by 0.3 meq/kg.

The p values comparing slopes of the treatments from the GLM are indicated in table 3.

Table 3

p values of the differences in lipolysis slope across treatments listed in figure 5 as measured by the GLM

	Control		
62 MPa 15C	<0.001	**62 MPa 15C**	
7 MPa 40C	<0.001	0.240	**7 MPa 40C**
62 MPa 40C	0.086	<0.001	<0.001

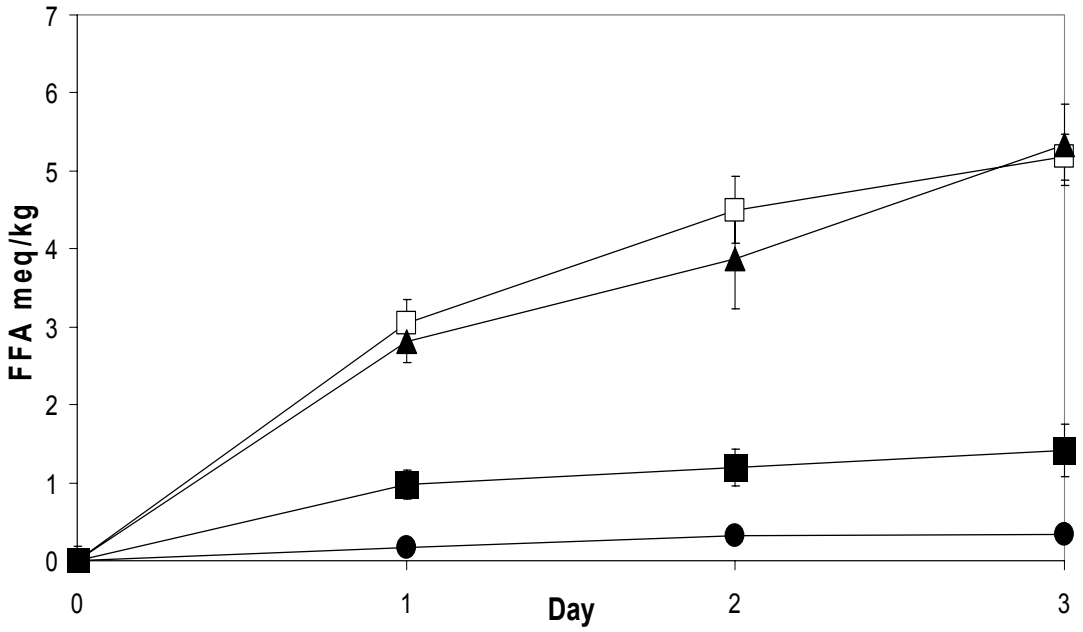

Figure 6

Mean (n=2) increase in FFA content of raw whole milk after DP CO_2 at 62 MPa 40°(—■—), 7 MPa 40°C(—▲—), 62 MPa 15°C (—□—), and an untreated control (—●—)

The results in figure 5 indicate a complex effect of the processing pressure and temperature on the rate of lipolysis. The low rate of lipolysis in the 62 MPa 40°C treatment may indicate that increased pressures add to the thermal inactivation of lipolytic enzymes. The lower pressure, 7 MPa, at 40°C may have been insufficient to affect the temperature of inactivation. Residual activity, coupled with an increased surface area may have caused the large FFA release. The lack of inactivation at 62 MPa and 15°C is an indication that treatment pressure was not solely responsible for the decrease in rates of lipolysis. The net effect is that a minimal combination of temperature and pressure are required to inhibit lipolysis.

The sensory threshold for FFA in milk has been found to be 0.327 meq FFA/kg milk (Santos 2003). The levels of FFA produced in this experiment are in the range of 5 meq FFA or more, which would tend to be consistent to what would be found in aged cheese (Licitra 2000). With the exception of the control sample, all treatments in figure 4 rapidly attained a FFA content that would be considered a sensorial defect in fluid milk.

Balaban's (1991) work on pectinesterase in orange juice found that the enzyme could be inactivated with supercritical CO_2 at temperatures below what is necessary for thermal inactivation. Reduced thermal inactivation is similar to what was found with the treatment of milk at 62 MPa and 40°C. This experiment showed that the enzymes responsible for lipolytic activity in milk are being inactivated at a temperature lower than the 60°C required for thermal treatment alone. Balaban also found that higher temperatures and pressures accounted for more inactivation, which would explain why the highest level of inactivation was at the highest temperature and pressure.

The data in table 4 shows the D value, which is the time in minutes which it takes for the enzyme activity to drop 90%, for each treatment.

Table 4

D values of pectinesterase at 3 temperatures, with and without the addition of CO_2 (Balaban 1991)

	40°	55°	60°
D value without CO_2	2673.8	141.8	56.6
D value with CO_2	104.6	20.9	12.1

When DP CO_2 was applied to whole milk, higher process temperatures decreased lipase activity at a given temperature (figure 6).

All treatments showed a larger increase in FFA than the control (mean day 3 ADV = 1.011). The 15° treatment showed the greatest increase in lipolysis (mean day 3 ADV = 7.043) followed by 22° (mean day 3 ADV = 5.408), 32° (mean day 3 ADV = 2.042), and 40° (mean day 3 ADV = 1.675).

All treatment temperatures were significantly different from one another except the control and 40°C and 32°C. P values comparing slopes of the treatments from the GLM are indicated in table 5.

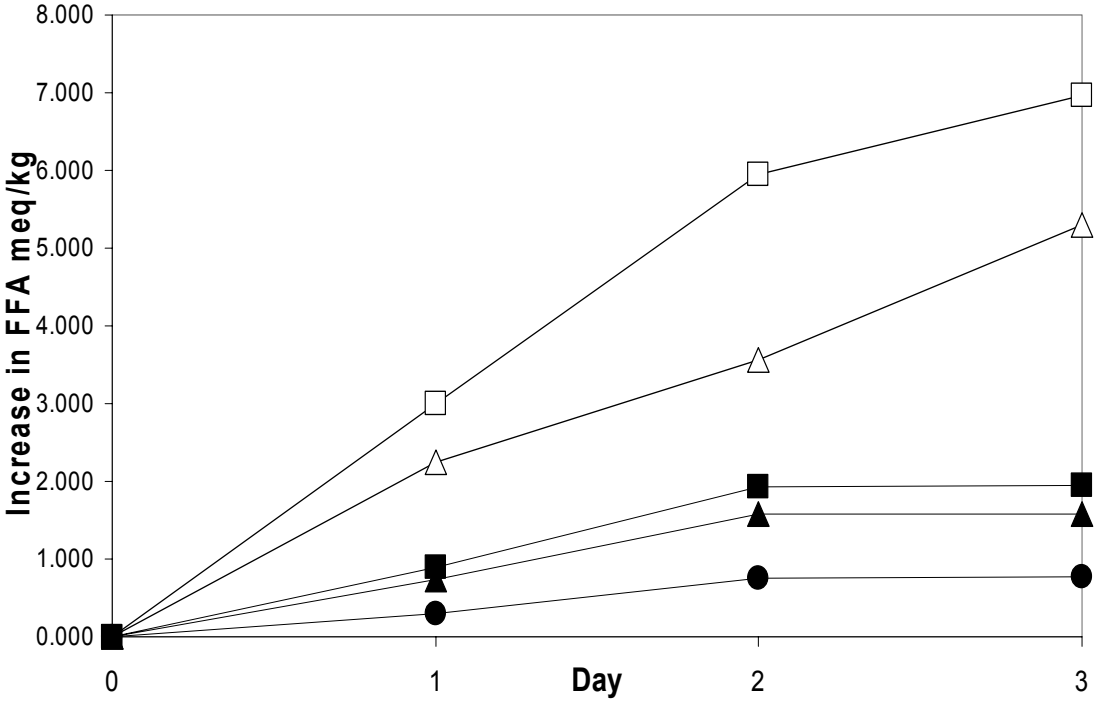

Figure 7

Mean (n=2) increase of FFA in whole milk inline carbonated at 62 MPa at 15°C (—□—), 22°C (—△—), 32°C (—■—), 40°C (—▲—), and an untreated control (—●—) after 3 days of 25° storage.

Table 5

p values of the differences in lipolysis slope across treatments listed in figure 6 as measured by the GLM

	Control			
15°C	<.001	**15°C**		
22°C	<.001	.001	**22°C**	
32.5°C	.015	<.001	<.001	**32.5°C**
40°C	.079	<.001	<.001	.446

All process temperatures show an increase in lipolysis. The general trend observed was a decrease in the rate of lipolysis with an increase in processing temperature. At 40°C, however, the difference between CO_2 treatment and the control is not significantly different at a 95% confidence interval. This further demonstrates that DP CO_2 treatment make enzymes more heat labile, not inactivate them directly. Increased efficacy of DP CO_2 treatment at higher temperatures also explains why the highest level of inactivation was at the highest temperature.

When milk was pressurized to the same levels used in figure 5 without CO_2, the treatment did not have a significant inhibitory effect on the rate of lipolysis (figure 7).

All treatments show a significant increase in lipolytic rates compared to the control. However, none of the individual temperature treatments were significantly different from each other.

At ambient temperatures, enzymes do not begin to be inactivated unless pressures reach the 207 – 608 MPa range (Hendrickx, and Knorr 1999). This would explain the lack of inactivation observed in figure 7. However, inactivation pressure may be reduced by external sources such as

pH (Balny and Masson 1993). The inactivation at higher temperatures in DP CO_2 treatments (figure 6) may have been caused by pressure acting synergistically with a drop in pH. The hydration reactions that result when CO_2 is dissolved in an aqueous medium with a pH of less than 8.0 cause an acidification of the milk.

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \text{ (Dixon, 1989)}$$

The increase in the rate of hydrolysis for all whole milk lipolysis experiments is likely caused by a change in the fat globule size of the milk. The increased surface area caused by homogenization allowed the lipoprotein lipase more access to its substrate. Therefore, the high FFA values observed were due to this homogenization of the fat.

To eliminate the effect of processing on fat globule size, raw skim was used as a substrate for DP CO_2 processing. After treatment, pasteurized, unhomogenized heavy cream was added to the skim to serve as a uniform substrate for lipolysis. This treatment of raw skim milk resulted in a decrease in FFA over time (figure 8).

The rate of lipolysis in the 62 MPa 15°C sample was significantly larger than an untreated control, and smaller in the 62 MPa 40° treatment. The GLM slope comparisons are shown below (table 6).

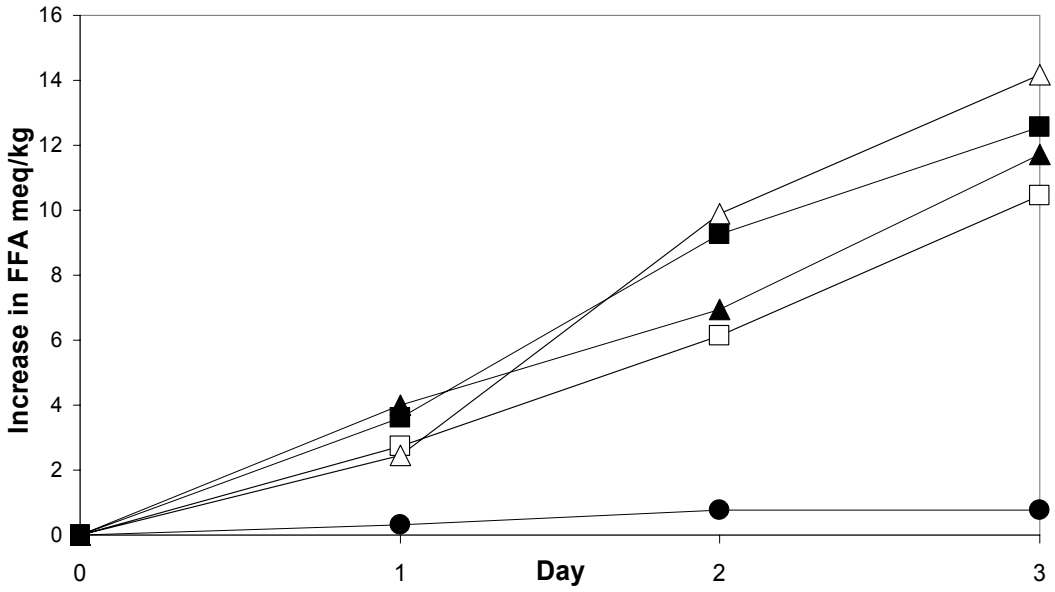

Figure 8

Mean increase of FFA in whole milk pressurized to 62 MPa at 15°C (—□—), 22°C (—△—), 32°C (—■—), 40°C (—▲—), and an uncarbonated control (—●—) after 3 days of 25° storage.

Table 6

p values of the differences in lipolysis slope across treatments listed in figure 7 as measured by the GLM

	Control			
62 MPa 15°	<.001	**62 MPa 15°**		
62 MPa 22°	.083	.002	**62 MPa 22°**	
62 MPa 32°	.214	<.001	.004	**62 MPa 32°**
62 MPa 40°	.037	<.001	<.001	.380

The most noticeable change in the results of this experiment with skim compared to the results of the previous ones using whole milk (figures 5-7) is the scale of the inactivation. While previous lipolytic experiments with whole milk had FFA values as high as 14 meq/kg milk, all values in this experiment were all below 1 meq regardless of treatment. The crucial change between the experiments shown in figure 7 and those in figures 4-6 was the size of the milkfat particle.

Although this process was not designed to homogenize the fat, the results obtained in previous experiments are similar to the results Hayes (Hayes, McSweeney et al. 2002) obtained by homogenizing raw whole milk. By protecting the fat

globule by withholding fat from the high shear in the DP CO_2 process, we do not observe the large increase in FFA associated with homogenization. The significantly lower FFA release in the 40° treatment in figure 7 is likely due to the inactivation of the enzyme caused by the DP CO_2 inactivation observed earlier. The results of this experiment are clearer because the rates of lipolysis are unclouded by the homogenization of the fat. Pre-

homogenization failed to provide a protective effect on the fat globule size of

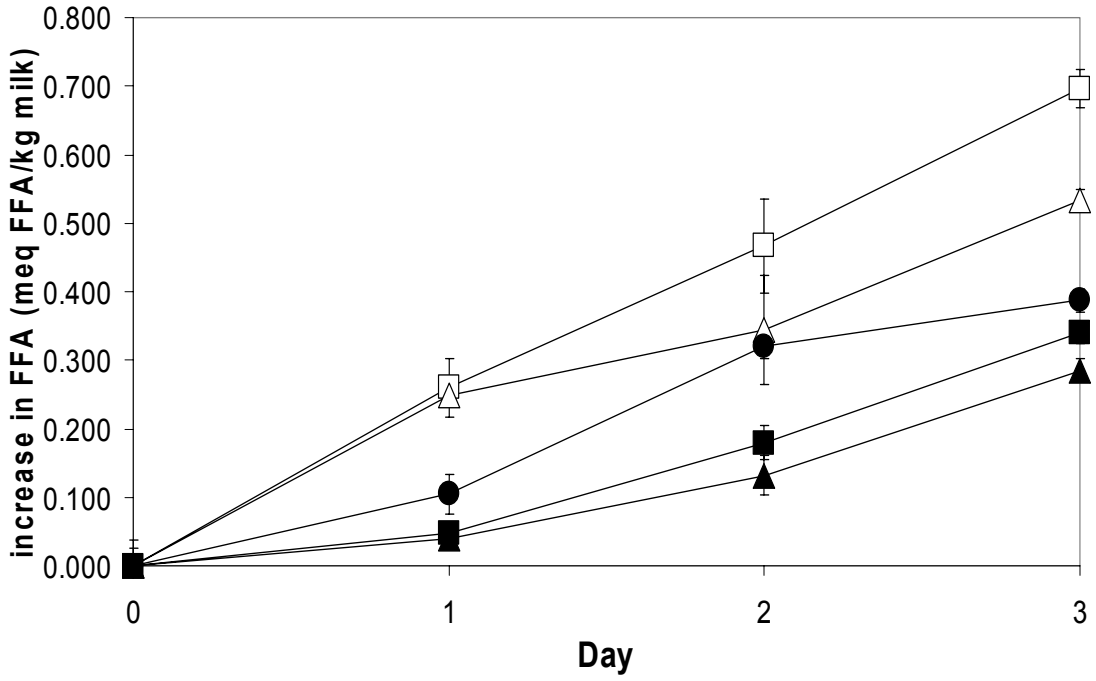

Figure 9

Mean increase of FFA in skim milk carbonated at 62 MPa at 15°C (—□—), 22°C (—△—), 32°C (—■—), 40°C (—▲—), and an uncarbonated control (—●—) after 3 days of 25° storage. Pasteurized, unhomogenized cream was added as a fat substrate after DP CO_2 processing.

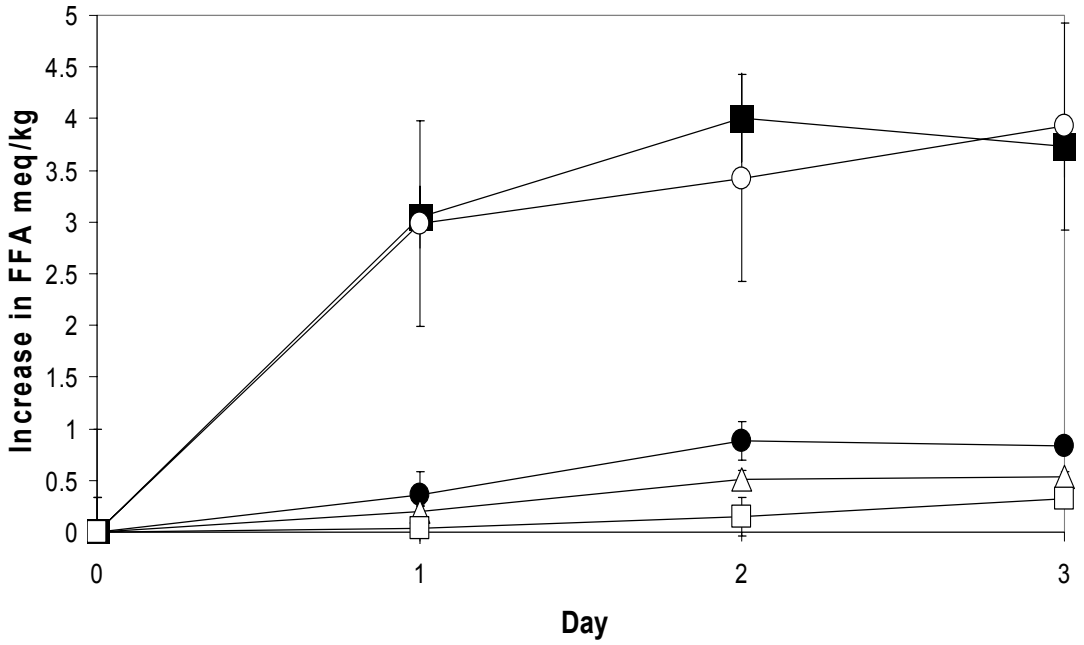

Figure 10

Mean increase of FFA in whole milk homogenized at 50° (—■—), homogenized at 50° and run through the Praxair unit at no pressure (—○—), 50° 7 MPa DP CO_2 (—△—), 50° 62 MPa DP CO_2 (—□—), and an untreated control (—●—), after 3 days of 25° storage.

raw milk, and all samples had an altered PSD (data not shown). This process was done at higher temperatures than previous experiments (50°C compared to 40°C) so its inhibitive effects on lipolytic enzymes (figure 9) were predicted.

The lipolysis observed in the homogenized milk run through the system without carbon dioxide (—○—), was similar to the homogenized control (—■—). In both treatments, the FFA content of the milk increased approximately 3.75 meq, indicating that the Praxair unit alone was not causing enzyme activation or inactivation. The FFA content of milk pressurized with CO_2 at 7 and 62 MPa increased to lower levels than the unprocessed controls suggesting that enzyme activity was inactivated.

This experiment further demonstrates that addition of carbon dioxide to a process flow decreases the temperature at which lipase enzymes are inactivated. All treatments except the control were done at 50°C, which is not normally sufficient to inactivate lipase enzymes as demonstrated by the high levels of FFA in the uncarbonated, homogenized control. The inactivation caused by the addition of carbon dioxide at 7 and 62 MPa show that DP CO_2 decreased the temperature necessary to inactivate lipase enzymes.

4.2.2 Particle Size

DP CO_2 treatment at 62 MPa changed the particle size distribution (PSD) of raw, whole milk compared to the untreated controls in all treated samples (figure 10).

Due to limitations in the Mastersizer™ PSD detector, particles larger than 100 μm were not able to be seen. A large peak was observed at the upper range of the detectable particle sizes at lower processing temperatures (15° and 22°C). This is likely the formation of large fat granules similar to what

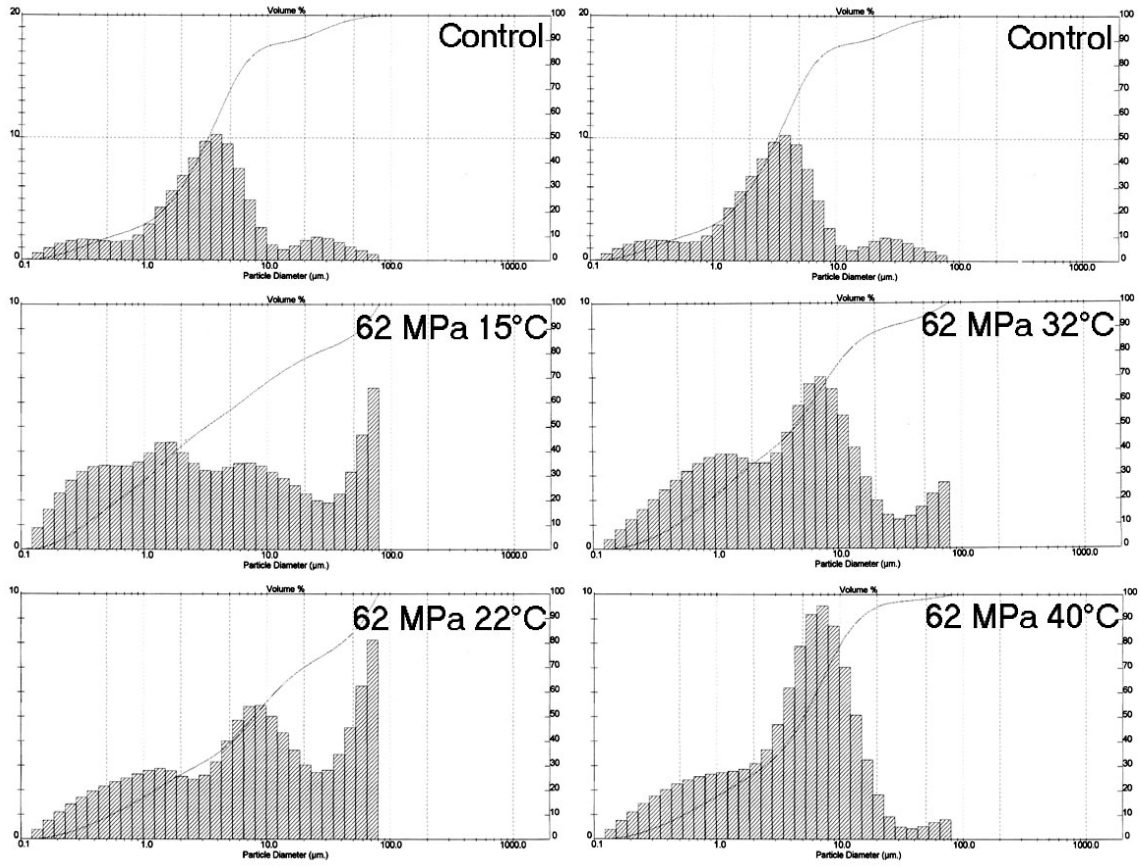

Figure 11

Particle Size Distribution of raw whole milk untreated and processed with DP CO_2 at 62 MPa at 15°C, 22°C, 32°C, and 40°C

would be found in butter. The formation of these particles at lower temperatures coincide with the commercial manufacture of butter at temperatures around 15°C (Tetrapak). This is in contrast with the 60°C temperatures typically used in homogenization, which may explain the more uniform distribution at the 40°C treatment.

The peak in the main mode of the control sample was at about 4 μm. When CO_2 was applied, a new peak emerged in the distributions at about 8μm, which became larger as temperature increased. The formation of a larger peak suggests that it is not composed entirely of individual fat globules, and may contain agglomerated caseins. The change in the PSD indicates a modification to the fat when milk was treated with CO_2 and pressure. This new peak at 8 μm (figure 10) may be explained by a homogenization of the fat (disappearance of the 4 μm peak in the control) and the creation of a fat protein complex with a slightly larger particle size (the 8 μm peak).

The removal of fat from the processing run provided clearer images of the denaturing effect of DP CO_2 when compared to a control skim (figure 11).

All treatment conditions had substantial changes in the particle size distribution of the skim milk. Treatments at 62 MPa had a smaller volume mean particle diameter than the 7 MPa treatments, likely caused by the breakup of larger sized protein agglomerates after a greater pressure drop. Because the particle size changed between the control and processed samples, it is difficult to say whether differences in the rates of lipolysis or proteolysis were caused by an effect on the enzymes or change in substrate.

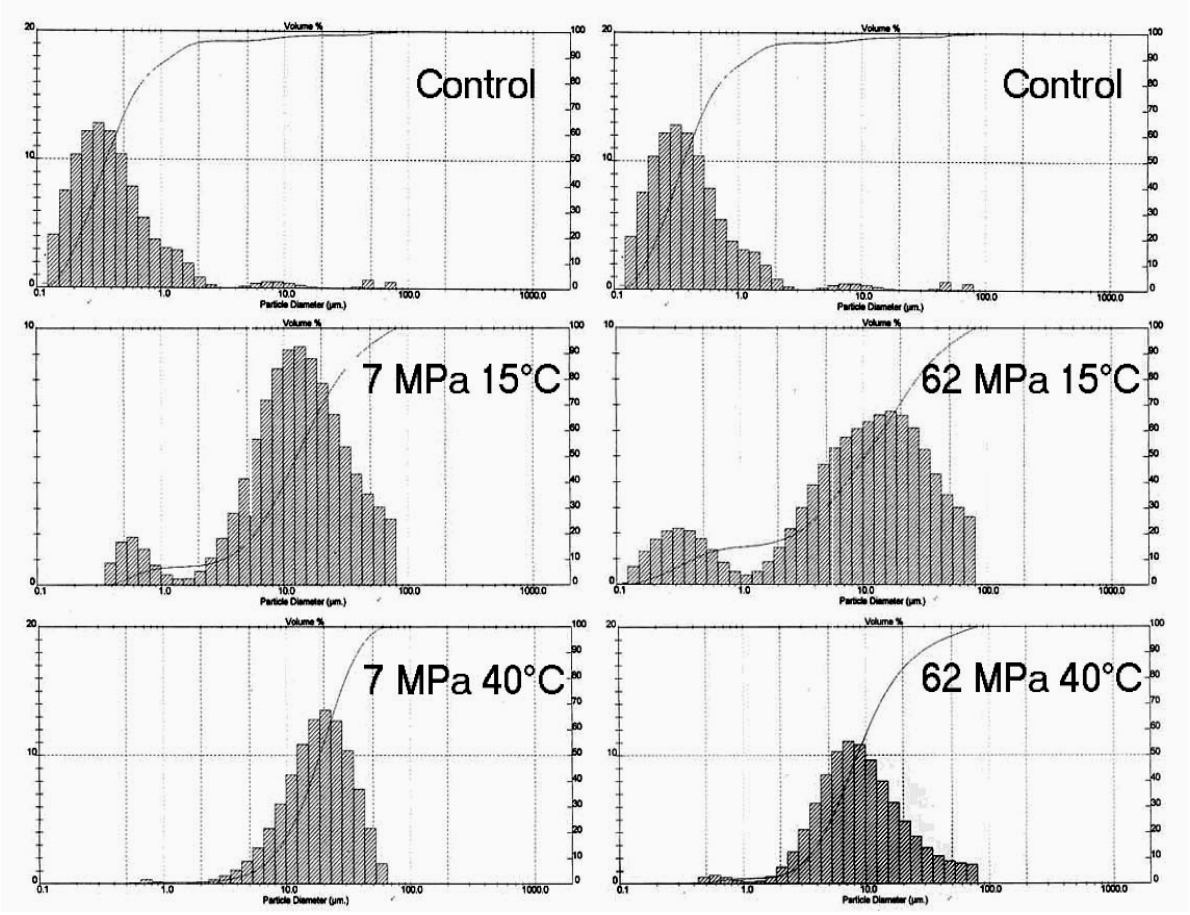

Figure 12

PSD of raw skim milk untreated and processed at 7 MPa 15°C, 7 MPa 40°C, 62 MPa 15°C, and 62 MPa 40°C. Control PSD is repeated for comparison.

4.2.3 Proteolysis

DP CO_2 processing of raw, whole milk decreased the rate of proteolytic activity over that of an untreated control (figure 12).

Casein as a percentage of total nitrogen (CPTN) decreased over 72 hours from approximately 78.4% to approximately 75% for all DP CO_2 treatments. This decrease was smaller than the change in control milk, which decreased from 78.5% to 72.0%.

These two experiments show that through the range of pressures and temperatures experimented on, all treatments had lower proteolytic rates than the control. The statistical test obtained from the GLM shows that the control category is significantly lower than the other 3 processes (P<.001). All other comparisons are not statistically significant (table 7).

Table 7

p values of the differences in proteolysis slopes across treatments listed in figure 12 as measured by the GLM

	Control		
62 MPa 15C	<.001	**62 MPa 15C**	
7 MPa 40C	<.001	.746	**7 MPa 40C**
62 MPa 40C	<.001	.147	.166

This experiment suggested that proteolysis is inhibited across the range of temperatures or pressures tested. Plasmin, the primary enzyme responsible for proteolysis is extremely heat stable, and would not be thermally inactivated at these temperatures (St-Gelais, Champagne et al. 1997). Therefore this process provided a novel approach to limit the rate of proteolysis in whole milk.

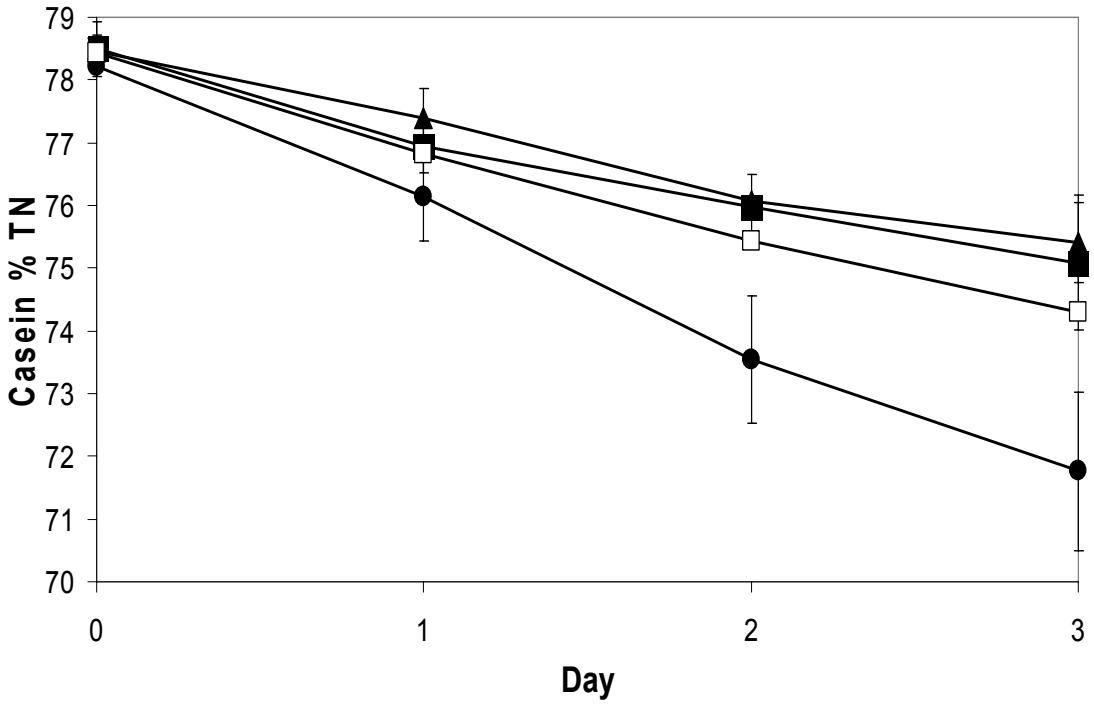

Figure 13

CPTN of raw whole milk after DP CO_2 at 62 MPa 40°(—■—), 7 MPa 40°C(—▲—), 62 MPa 15°C (—□—), and an untreated control (—●—) after 3 days of storage at 25°C

A phenomenon observed in homogenization (Barbano et. al unpublished data),(Hayes 2003)may explain the decrease in the rate of proteolysis after processing. Barbano (personal communication) theorized this proteolytic inhibition after homogenization was due to the movement of a portion of the casein protein from the serum phase to of the milk to the newly formed milkfat globule membrane. This movement of the protein effectively inhibits the action of enzymes by physically isolating the substrate thus giving the appearance of a decrease in proteolysis rate. Hayes (2002) theorized that this inactivation was due to some combination of the heat and physical forces experienced by the milk during homogenization.

When fat was eliminated from the process by using skim milk, DP CO_2 processing was still inhibitory to the rate of proteolysis (figure 13).

Casein as a percentage of total nitrogen decreased over time from approximately 77.4% to 72.9% (7 MPa 40°C), 68.8% (7 MPa 15°C), 68.1% (62 MPa 40°C) and 71.9% (62 MPa 15°C). The control treatment decreased in C%TN significantly more to an average value of 64.0% (table 8).

Table 8

p values of the differences in proteolysis slopes across treatments listed in figure 13 as measured by the GLM

	Control			
62 MPa 15C	<.001	**62 MPa 15C**		
62 MPa 40C	<.001	.804	**62 MPa 40C**	
7 MPa 15C	<.001	.011	.020	**7 MPa 15C**
7 MPa 40C	<.001	.002	.004	.523

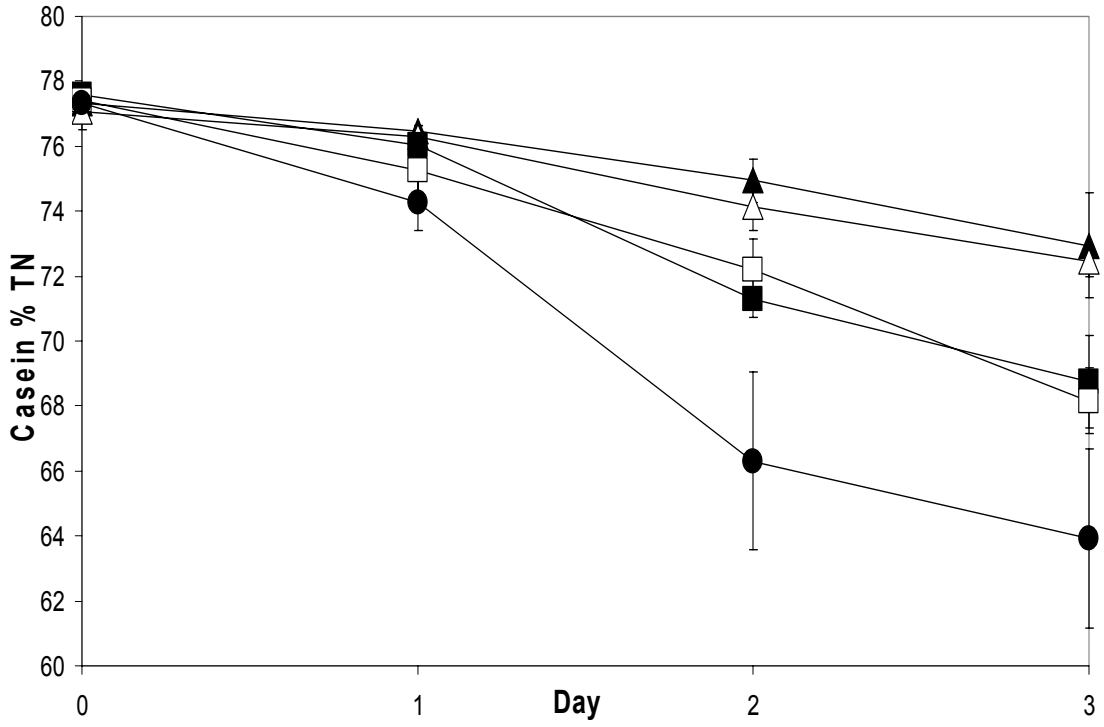

Figure 14

CPTN of raw skim milk after DP CO_2 processing at 62 MPa 40°(—■—), 62 MPa 15°C (—□—), 7 MPa 40°C(—▲—), 7 MPa 15°C (—△—) and an untreated control (—●—) after 3 days of storage at 25°C

Unlike the series of lipolysis experiments where higher temperatures, appeared to be the factor responsible for the inactivation of enzyme activity, the effect of temperature was minimal. Temperature did not have a significant effect on proteolysis, but the pressure of treatments was significant. Milk treated at 7 MPa had a significantly lower rate of proteolysis than the 62 MPa treatments at all temperatures. This is probably caused by more than one force acting in tandem. The inhibition caused by the DP CO_2 process, likely to be more severe in the 62 MPa treatments, is interacting with the particle size change of the treatments.

Treatments at 7 MPa produced a larger particle than when processed at 62 MPa (volume mean diameters of 19.3μm and 14.6μm respectively). With less surface area for the enzyme to work on in the 7 MPa treatment, the rate of proteolysis was effectively reduced.

4.3 Pre-carbonation Experiments

4.3.1 Particle Size

CO_2 was added to samples before pressurization in an attempt to reduce the amount of precipitated protein. At 62 MPa increased processing temperatures above 15°C resulted in milks with higher amounts of denatured proteins (figure 14). However, a procedure of pre-carbonation of milk and processing at 15°C was found to leave the PSD of the sample unchanged. Therefore, 15°C processing was used because this temperature treatment did not change the PSD of milk and allowed for observing the effect of DP CO_2 on enzyme activity without the presence of denatured caseins.

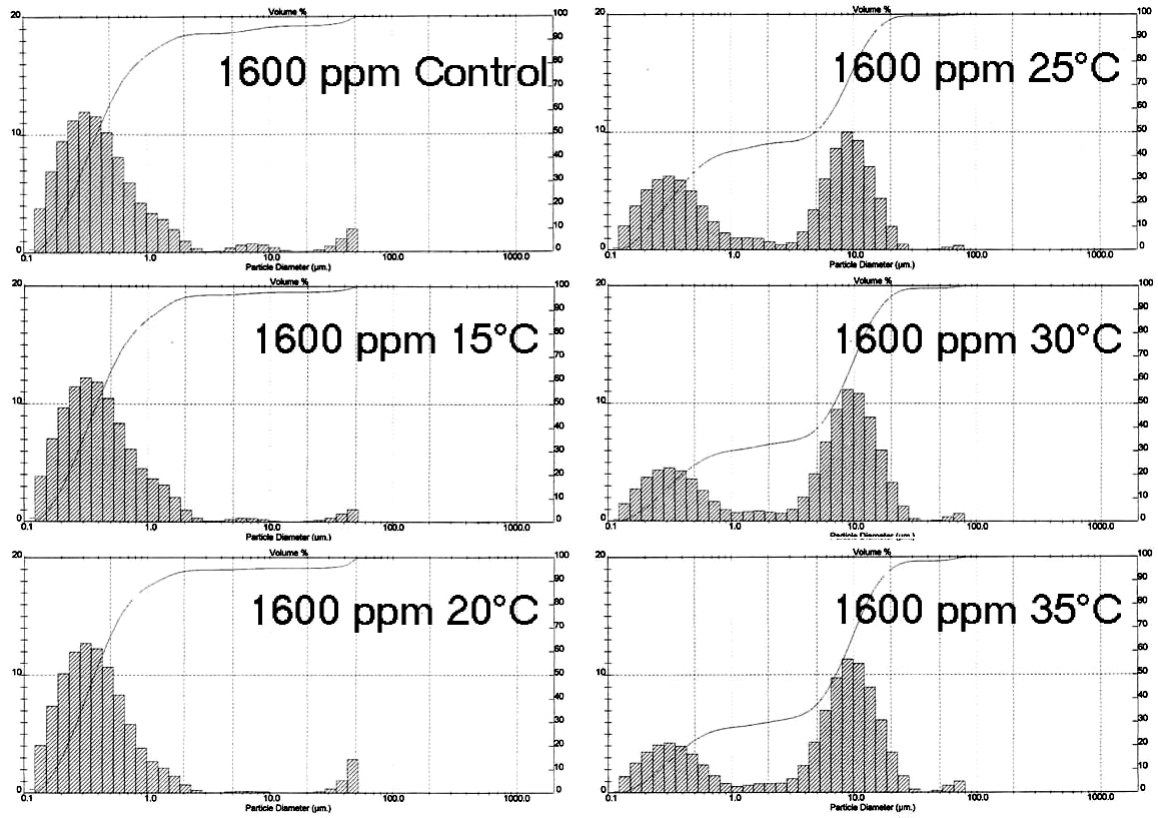

Figure 15

PSD of raw skim milk and raw skim milk with 1600 ppm CO_2 and pressurized to 62 MPa at 15°C, 22°C, 35°C, 40°C.

4.3.2 Lipolysis

At 15°C, the application of DP CO_2 had limited effect of changing the release of FFA over time (figure 15).

All treatments except the control increased in FFA content approximately 0.450 meq/kg. These treatments are consistent with the levels of released FFA from previous treatments of skim with cream added back (figure 8).

The only significant effect found was that the control treatment was significantly more lipolytic than all other treatments (table 9). One of the three replicates included a high outlier on day 3 of the control. There was no change in significance when the statistics were run with and without this point. Median values were plotted (figure 15) to graphically remove the presence of this outlier. Error bars in figure 15 are calculated without this point.

Whatever the reason for this increased lipolysis, it is not observed in any of the other non carbonated samples or those treatments with carbonation but not pressure. Although there was an inhibition of lipolysis with DP CO_2 treatment in previous experiments, it was found at higher temperatures of processing. Treating milk at 15°C, which does not change the particle size distribution of the sample, produced no noticeable inhibition above the effect of pressure or carbonation without pressure alone. While the temperature of inactivation may be reduced with DP CO_2 treatment, it is likely that 15°C is not sufficient to thermally inactivate this enzyme.

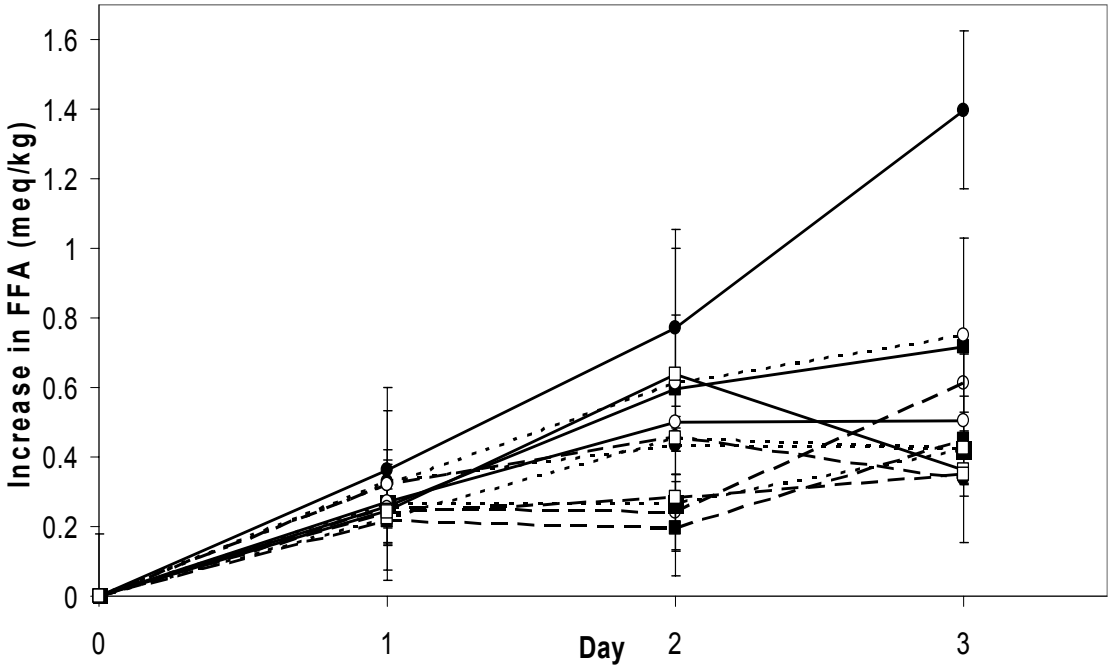

Figure 16

Median increase in FFA content of 11 treatments of milk and a control for 3 days of incubation at 25°C: 0 ppm no pressure (—●—), 0 ppm 7 MPa (▪▪▪●▪▪▪), 0 ppm 62 MPa (- - ●- -), 800 ppm no pressure (—■—), 800 ppm 7 MPa (▪▪▪■▪▪▪), 800 ppm 62 MPa (- - ■- -) 1600 ppm no pressure (—○—), 1600 ppm 7 MPa (▪▪▪○▪▪▪), 1600 ppm 62 MPa (- - ○- -), 2400 ppm no pressure (—□—), 2400 ppm 7 MPa (▪▪▪□▪▪▪), 2400 ppm 62 MPa (- - □- -)

Table 9

p Values of the difference in slope of FFA between pre-carbonated skim treatments in figure 15 as measured by the GLM

	0 C									
0 1k	0	**0 1k**								
0 9k	0	0.659	**0 9k**							
800 C	0	0.228	0.101	**800 C**						
800 1k	0	0.792	0.482	0.346	**800 1k**					
800 9k	0	0.749	0.447	0.376	0.955	**800 9k**				
1600C	0	0.668	0.385	0.437	0.868	0.913	**1600C**			
1600 1k	0	0.482	0.253	0.615	0.659	0.701	0.784	**1600 1k**		
1600 9k	0	0.522	0.281	0.571	0.706	0.749	0.833	0.949	**1600 9k**	
2400 C	0	0.943	0.609	0.257	0.847	0.803	0.72	0.527	0.569	**2400 C**
2400 1k	0	0.91	0.58	0.274	0.88	0.835	0.751	0.554	0.598	0.967
2400 9k	0	892	0.76	0.18	0.69	0.648	0.572	0.401	0.438	0.836

4.3.3 Proteolysis

Milk at 4 levels of carbonation pressure treated at 3 processing levels did not have a significant effect on the rate of proteolysis (figure 16).

The statistical evaluation of the data did not reveal a significant difference across all treatments (table 10). This lack of significance is in contrast to the change in activity found from the previous carbonation experiments.

The reason for the difference between in-line and pre-carbonation treatments may be due to the change in particle size. PSD readings from in-line carbonation experiments indicate a change in the structure of the protein. This structural effect may have caused proteolytic inhibition through the altering the substrate. It may be possible that the lower pH produced from in-line treatments have had an actual inhibition on the causative enzyme, however this is unlikely due to the high acid stability of the primary milk protease, plasmin (Kaminogawa 1972).

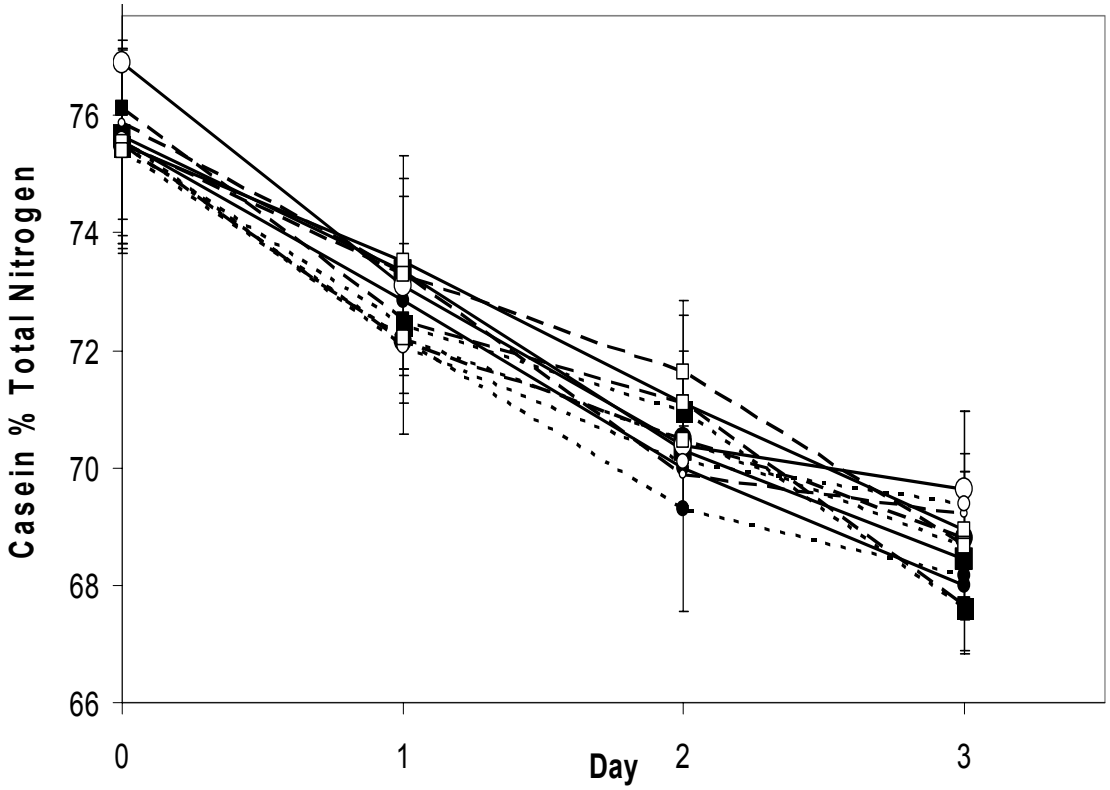

Figure 17

CPTN values of 11 treatments of skim milk and an untreated skim control for 3 days of incubation at 25°C: 0 ppm no pressure (—●—), 0 ppm 7 MPa (▪▪▪●▪▪▪), 0 ppm 62 MPa (- - ●- -), 800 ppm no pressure (—■—), 800 ppm 7 MPa (▪▪▪■▪▪▪), 800 ppm 62 MPa (- - ■- -) 1600 ppm no pressure (—○—), 1600 ppm 7 MPa (▪▪▪○▪▪▪), 1600 ppm 62 MPa (- - ○- -), 2400 ppm no pressure (—□—), 2400 ppm 7 MPa (▪▪▪□▪▪▪), 2400 ppm 62 MPa (- - □- -)

Table 10

p Values of the difference in slope of C%TN between treatments listed in figure 16 as measured by the GLM

	0 C										
0 1k	0.424	**0 1k**									
0 9k	0.418	0.992	**0 9k**								
800 C	0.669	0.709	0.702	**800 C**							
800 1k	0.745	0.635	0.628	0.919	**800 1k**						
800 9k	0.857	0.328	0.323	0.544	0.613	**800 9k**					
1600C	0.725	0.654	0.647	0.94	0.979	0.595	**1600C**				
1600 1k	0.799	0.585	0.579	0.863	0.943	0.664	0.922	**1600 1k**			
1600 9k	0.594	0.183	0.18	0.337	0.391	0.724	0.376	0.431	**1600 9k**		
2400 C	0.54	0.851	0.843	0.853	0.774	0.429	0.795	0.72	0.253	**2400 C**	
2400 1k	0.695	0.683	0.676	0.971	0.948	0.568	0.969	0.891	0.355	0.825	**2400 1k**
2400 9k	0.65	0.682	0.891	0.926	0.739	0.581	0.699	0.906	0.392	0.59	0.741

4.3 Conclusion

The application of carbon dioxide shows promise in the realm of non-thermal processing. In-line addition of CO_2 was able to significantly effect the chemistry of milk when processed at temperatures between 15°C and 40°C and at pressures between 7 and 62 MPa.

Processing whole milk at 40°C inactivated lipases at temperatures lower than thermal processing alone. Processing raw whole milk at 15°C was unable to inactivate lipolytic enzymes. The failure to inactivate lipases coupled with the increase in fat surface area from homogenization of the fat led to high levels of FFA in the milk after 3 days.

All in-line treatments were accompanied by a change in the PSD in both skim and whole milk. Treatments with an altered PSD also showed an inhibition of the proteolytic activity of the milk, likely caused by the change in protein structure of the milk.

Pre-carbonated skim milk treated at 15°C at the same pressures had no noticeable change in the PSD. At these treatment parameters there was also no appreciable change in the lipolytic or proteolytic activity of the milk from the Dense Phase CO_2.

REFERENCES

Andrews, A. T., M. Anderson, et al. (1987). "A Study of the Heat Stabilities of a Number of Indigenous Milk Enzymes." Journal of Dairy Research **54**(2): 237-246.

Arreola, A. G., M. O. Balaban, et al. (1991). "Effect of Supercritical Carbon-Dioxide on Microbial-Populations in Single Strength Orange Juice." Journal of Food Quality **14**(4): 275-284.

Arroyo, G., P. D. Sanz, et al. (1997). "Effect of high pressure on the reduction of microbial populations in vegetables." Journal of Applied Microbiology **82**(6): 735-742.

Balaban, M. O., A. G. Arreola, et al. (1991). "Enzyme Inactivation in Supercritical Carbon-Dioxide Conditions." Abstracts of Papers of the American Chemical Society **202**: 28-AGFD.

Ballestra, P., A. A. Dasilva, et al. (1996). "Inactivation of Escherichia coli by carbon dioxide under pressure." Journal of Food Science **61**(4): 829-&.

Balny, C. and P. Masson (1993). "Effects of High-Pressure on Proteins." Food Reviews International **9**(4): 611-628.

Calvo, M. M. and E. Balcones (2001). "Inactivation of microorganisms and changes of proteins during treatment of milk with subcritical carbon dioxide." Milchwissenschaft-Milk Science International **56**(7): 366-369.

Chen, J. H. and J. H. Hotchkiss (1991). "Effect of Dissolved Carbon-Dioxide on the Growth of Psychrotrophic Organisms in Cottage Cheese." Journal of Dairy Science **74**(9): 2941-2945.

Corwin, H. and T. H. Shellhammer (2002). "Combined carbon dioxide and high pressure inactivation of pectin methylesterase, polyphenol oxidase, Lactobacillus plantarum and Escherichia coli." Journal of Food Science **67**(2): 697-701.

de Ancos, B., S. Sgroppo, et al. (2002). "Possible nutritional and health-related value promotion in orange juice preserved by high-pressure treatment." Journal of the Science of Food and Agriculture **82**(8): 790-796.

Dillow, A. K., F. Dehghani, et al. (1999). "Bacterial inactivation by using near- and supercritical carbon dioxide." Proceedings of the National Academy of Sciences of the United States of America **96**(18): 10344-10348.

Dixon, N. M. and D. B. Kell (1989). "The Inhibition by Co2 of the Growth and Metabolism of Microorganisms." Journal of Applied Bacteriology **67**(2): 109-136.

Enomoto, A., K. Nakamura, et al. (1997). "Lethal effect of high-pressure carbon dioxide on a bacterial spore." Journal of Fermentation and Bioengineering **83**(3): 305-307.

Enomoto, A., K. Nakamura, et al. (1997). "Inactivation of food microorganisms by high-pressure carbon dioxide treatment with or without explosive decompression." Bioscience Biotechnology and Biochemistry **61**(7): 1133-1137.

Erkmen, O. (2000). "Antimicrobial effect of pressurised carbon dioxide on Enterococcus faecalis in physiological saline and foods." Journal of the Science of Food and Agriculture **80**(4): 465-470.

Erkmen, O. (2000). "Effect of carbon dioxide pressure on Listeria monocytogenes in physiological saline and foods." Food Microbiology **17**(6): 589-596.

Erkmen, O. (2000). "Inactivation of Salmonella typhimurium by high pressure carbon dioxide." Food Microbiology **17**(2): 225-232.

Erkmen, O. (2001). "Effects of high-pressure carbon dioxide on Escherichia coli in nutrient broth and milk." International Journal of Food Microbiology **65**(1-2): 131-135.

Erkmen, O. (2001). "Kinetic analysis of Listeria monocytogenes inactivation by high pressure carbon dioxide." Journal of Food Engineering **47**(1): 7-10.

Erkmen, O. (2001). "Mathematical modeling of Escherichia coli inactivation under high-pressure carbon dioxide." Journal of Bioscience and Bioengineering **92**(1): 39-43.

Erkmen, O. and H. Karaman (2001). "Kinetic studies on the high pressure carbon dioxide inactivation of Salmonella typhimurium." Journal of Food Engineering **50**(1): 25-28.

FDA (1999). Grade "A" Pasteurized Milk Ordinance. Department of Health and Human Services. **Washington, DC**.

Fennema, Ed. (1996). Food Chemistry. Food Science and Technology. New York, NY, Marcel Dekker, Inc.

Fox, P. F., Ed. (1992). Advanced dairy chemistry.

Garcia, A. F., P. Butz, et al. (2001). "Antioxidative capacity, nutrient content and sensory quality of orange juice and an orange-lemon-carrot juice product after high pressure treatment and storage in different packaging." European Food Research and Technology **213**(4-5): 290-296.

Haas, G. J., H. E. Prescott, et al. (1989). "Inactivation of Microorganisms by Carbon-Dioxide under Pressure." Journal of Food Safety **9**(4): 253-265.

Hayes, M. G., A. L. Kelly, et al. (2002). "The influence of native and heat-denatured whey proteins on enzyme activity. 2. Chymosin." Milchwissenschaft-Milk Science International **57**(5): 264-267.

Hayes, M. G., P. L. H. McSweeney, et al. (2002). "The influence of native and heat-denatured whey proteins on enzyme activity. 1. Plasmin." Milchwissenschaft-Milk Science International **57**(4): 208-211.

Hendrickx, M., L. Ludikhuyze, et al. (1998). "Effects of high pressure on enzymes related to food quality." Trends in Food Science & Technology **9**(5): 197-203.

Hill, W. S., M. R. Marshall, et al. (2000). "Pathogen reduction in fruit juices with a continuous flow high pressure carbon dioxide system." Abstracts of Papers of the American Chemical Society **220**: 38-AGFD.

Hite B. N., G. N. J., WEAKLY C.E., (1899). "The effect of pressure in the preservation of milk." Bull. West Virginia Agr. Exp. Sta. **3**(15): 58.

Hofland, G. W., M. van Es, et al. (1999). "Isoelectric precipitation of casein using high-pressure CO2." Industrial & Engineering Chemistry Research **38**(12): 4919-4927.

Hong, S. I. and Y. R. Pyun (1999). "Inactivation kinetics of Lactobacillus plantarum by high pressure carbon dioxide." Journal of Food Science **64**(4): 728-733.

Hong, S. I. and Y. R. Pyun (2001). "Membrane damage and enzyme inactivation of Lactobacillus plantarum by high pressure CO2 treatment." International Journal of Food Microbiology **63**(1-2): 19-28.

Ishikawa, H., M. Shimoda, et al. (1996). "Inactivation of enzymes and decomposition of alpha-helix structure by supercritical carbon dioxide microbubble method." Journal of Agricultural and Food Chemistry **44**(9): 2646-2649.

Iwatsuki, K., Y. Mizota, et al. (2000). "Effect of pasteurization temperature on the sensory characteristics of UHT processed milk - Studies on the sensory characteristics and physicochemical properties of UHT processed milk Part III." Journal of the Japanese Society for Food Science and Technology-Nippon Shokuhin Kagaku Kogaku Kaishi **47**(7): 538-543.

Jordan, P. J., K. Lay, et al. (1987). "Casein Precipitation Using High-Pressure Carbon-Dioxide." New Zealand Journal of Dairy Science and Technology **22**(3): 247-256.

Kamihira, M., M. Taniguchi, et al. (1987). "Sterilization of Microorganisms with Supercritical Carbon- Dioxide." Agricultural and Biological Chemistry **51**(2): 407-412.

Kaminoga.S, Mizobuch.H, et al. (1972). "Comparison of Bovine Milk Protease with Plasmin." Agricultural and Biological Chemistry **36**(12): 2163-2167.

King, A. D. and C. W. Nagel (1967). "Growth Inhibition of a Pseudomonas by Carbon Dioxide." Journal of Food Science **32**(5): 575-&.

Knorr, D., M. Hendrickx, et al. (2002). "Preface - EUROCAFT 2001 special issue." Trends in Food Science & Technology **13**(9-10): 290-290.

Kumagai, H., C. Hata, et al. (1997). "CO2 sorption by microbial cells and sterilization by high- pressure CO2." Bioscience Biotechnology and Biochemistry **61**(6): 931-935.

Lin, H. M., E. C. Chan, et al. (1991). "Disintegration of Yeast-Cells by Pressurized Carbon-Dioxide." Biotechnology Progress **7**(3): 201-204.

Lin, H. M., Z. Y. Yang, et al. (1992). "An Improved Method for Disruption of Microbial-Cells with Pressurized Carbon-Dioxide." Biotechnology Progress **8**(2): 165-166.

Lin, H. M., Z. Y. Yang, et al. (1992). "Inactivation of Saccharomyces-Cerevisiae by Supercritical and Subcritical Carbon-Dioxide." Biotechnology Progress **8**(5): 458-461.

Lynch, J. M. and D. M. Barbano (1999). "Kjeldahl nitrogen analysis as a reference method for protein determination in dairy products." Journal of Aoac International **82**(6): 1389-1398.

Ma, Y., C. Ryan, et al. (2000). "Effects of somatic cell count on quality and shelf-life of pasteurized fluid milk." Journal of Dairy Science **83**(2): 264-274.

Nakamura, K., A. Enomoto, et al. (1994). "Disruption of Microbial-Cells by the Flash Discharge of High- Pressure Carbon-Dioxide." Bioscience Biotechnology and Biochemistry **58**(7): 1297-1301.

Park, S. J., J. I. Lee, et al. (2002). "Effects of a combined process of high-pressure carbon dioxide and high hydrostatic pressure on the quality of carrot juice." Journal of Food Science **67**(5): 1827-1834.

Rizvi, S. S. H., J. A. Daniels, et al. (1986). "Supercritical Fluid Extraction - Operating Principles and Food Applications." Food Technology **40**(7): 57-64.

Ruas-Madiedo, P., L. Alonso, et al. (2002). "Manufacture of Spanish hard cheeses from CO2-treated milk." Food Research International **35**(7): 681-690.

RuasMadiedo, P., J. C. BadaGancedo, et al. (1996). "Preservation of the microbiological and biochemical quality of raw milk by carbon dioxide addition: A pilot-scale study." Journal of Food Protection **59**(5): 502-508.

Schwimmer (1981). Source Book of Food Enzymology. Westport, Connecticut, AVI Publishing INC.

Scollard, P. G., T. P. Beresford, et al. (2000). "Barostability of milk plasmin activity." Lait **80**(6): 609-619.

Shimoda, M., J. Cocunubo-Castellanos, et al. (2001). "The influence of dissolved CO2 concentration on the death kinetics of Saccharomyces cerevisiae." Journal of Applied Microbiology **91**(2): 306-311.

Shimoda, M. and Y. Osajima (1998). "Non-heating inactivation of microorganisms and enzymes - Application of supercritical carbon dioxide micro bubble method to food industry." Journal of the Japanese Society for Food Science and Technology-Nippon Shokuhin Kagaku Kogaku Kaishi **45**(5): 334-339.

Shimoda, M., Y. Yamamoto, et al. (1998). "Antimicrobial effects of pressured carbon dioxide in a continuous flow system." Journal of Food Science **63**(4): 709-712.

Shipe, W. F., G. F. Senyk, et al. (1980). "Modified Copper Soap Solvent-Extraction Method for Measuring Free Fatty-Acids in Milk." Journal of Dairy Science **63**(2): 193-198.

Sierra, I., M. Prodanov, et al. (1996). "Vitamin stability and growth of psychrotrophic bacteria in refrigerated raw milk acidified with carbon dioxide." Journal of Food Protection **59**(12): 1305-1310.

Sousa, M. J., Y. Ardo, et al. (2001). "Advances in the study of proteolysis during cheese ripening." International Dairy Journal **11**(4-7): 327-345.

Spilimbergo, S., N. Elvassore, et al. (2002). "Microbial inactivation by high-pressure." Journal of Supercritical Fluids **22**(1): 55-63.

St-Gelais, D., C. P. Champagne, et al. (1997). "Production of cheddar cheese using milk acidified with carbon dioxide." Milchwissenschaft-Milk Science International **52**(11): 614-618.

Taniguchi, M., M. Kamihira, et al. (1987). "Effect of Treatment with Supercritical Carbon-Dioxide on Enzymatic-Activity." Agricultural and Biological Chemistry **51**(2): 593-594.

Tedjo, W., M. N. Eshtiaghi, et al. (2000). "Impact of supercritical carbon dioxide and high pressure on lipoxygenase and peroxidase activity." Journal of Food Science **65**(8): 1284-1287.

Tomasula, P. M., R. T. Boswell, et al. (1999). "Buffer properties of milk treated with high pressure carbon dioxide." Milchwissenschaft-Milk Science International **54**(12): 667-670.

Tomasula, P. M., J. C. Craig, et al. (1997). "A continuous process for casein production using high-pressure carbon dioxide." Journal of Food Engineering **33**(3-4): 405-419.

Tomasula, P. M., J. C. Craig, et al. (1995). "Preparation of Casein Using Carbon-Dioxide." Journal of Dairy Science **78**(3): 506-514.

Tomasula, P. M., J. C. Craig, et al. (1998). "Economic analysis of a continuous casein process using carbon dioxide as precipitant." Journal of Dairy Science **81**(12): 3331-3342.

Vandenberg, M. G. (1979). "Partial-Pressure of Carbon-Dioxide in Milk and Its Relation to the Freezing-Point." Netherlands Milk and Dairy Journal **33**(2-3): 91-111.

Wei, C. I., M. O. Balaban, et al. (1991). "Bacterial Effect of High-Pressure Co2 Treatment on Foods Spiked with Listeria or Salmonella." Journal of Food Protection **54**(3): 189-193.

Weibe (1940). "Solubility of Carbon Dioxide in Water at Various Temperatures from 12 to 40C and Pressures to 500 Atmospheres." J. Amer. Chem. Soc. **62**: 815-817.

Yuste, J., M. Capellas, et al. (2001). "High pressure processing for food safety and preservation: A review." Journal of Rapid Methods and Automation in Microbiology **9**(1): 1-10.