Heat Stability of Residual Milk Clotting Enzymes in Cheese Whey

James Winter Duersch

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HEAT STABILITY OF RESIDUAL MILK CLOTTING ENZYMES
IN CHEESE WHEY

by

James Winter Duersch

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Nutrition and Food Science

UTAH STATE UNIVERSITY
Logan, Utah

1976
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James Winter Duersch
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ABSTRACT

Heat Stability of Residual Milk Clotting Enzymes in Cheese Whey

by

James Winter Duersch, Master of Science

Utah State University, 1976

Major Professor: Dr. C. A. Ernstrom
Department: Nutrition and Food Science

Eliminating calcium chloride and replacing whole casein with k-casein eliminated clouding and sharpened diffusion boundaries in casein-agar gels used for the estimation of residual milk clotting enzymes in curd and whey. It also eliminated the need for a highly purified calcium-free agar. The substrate contained .54 percent k-casein, 3.6 percent sodium acetate, .73 percent bacto-agar and 95.13 percent water. The pH was adjusted to 5.9 with .1N hydrochloric acid.

Proteases derived from Mucor pusillus var Lindt, Mucor miehei and Endothia parasitica, as well as rennet, procine and bovine pepsins were used at recommended levels to set milk for Cheddar cheese manufacture. Whey samples from each lot were taken at draining and adjusted to pH 5.2, 5.6, 6.0, 6.2, 6.6 and 7.0 prior to being heated to 68.3°C, 71.1°C and 73.9°C for .25, .5, 1, 5, and 10 minutes. Enzyme activities were assayed before and after heating. Mucor miehei protease was the most heat stable at all pH values followed by Mucor pusillus protease, rennet, bovine pepsin, E. parasitica protease and porcine pepsin.
The heat stability of all enzymes except *E. parasitica* protease decreased with increasing pH, *E. parasitica* protease decreased with decreasing pH.

All enzymes were inactivated at the minimum heat treatment at pH 7.0 except *E. parasitica* protease which was most stable at that pH. At pH 5.2 *M. miehei* protease persisted after a 10 minute treatment at 73.9°C.
INTRODUCTION

It was estimated that 30.5 billion pounds of both sweet and acid whey were produced in 1973 by the cheese industry in the United States, with only 43 percent processed for further use (95). Disposal of whey has been an increasing concern to the cheese industry. Economical ways of disposal have been sought along with the hope that profitable methods for utilization may be developed. Increased concern about pollution has increased the cost of whey disposal. Due to this added expense the cheese industry has developed an interest in using this by-product as a means of making money instead of losing it. Development of many food products with whey as a constituent has resulted from this effort.

An important factor in utilization of whey has been the effect of residual milk clotting enzymes in processed whey products. The presence of these enzymes in food products containing milk has been reported to cause coagulation of the milk proteins and spoil the product. Infant formulas and dairy blends for ice cream mixes are products that contain whey, and that have been adversely affected by residual enzymes.

Until 1973 methods for quantitatively measuring low concentrations of milk clotting enzymes were sought with minor success. Holmes and Ernstrom (43) developed an assay involving the linear diffusion of milk clotting enzymes through a casein-agar diffusion substrate. They were able to measure rennet at concentrations as low as $1 \times 10^{-4}$ rennin units per milliliter. Use of whole casein and CaCl$_2$ in the substrate
frequently produced clouding in the diffusion tubes which made detection of the diffusion distances difficult. Also the use of a highly purified agar was required due to the sensitivity of the whole casein to calcium.

The purpose of this project was to find a method to eliminate clouding in the diffusion substrate and produce a sharp diffusion boundary. A further objective was the study of the heat stability of residual milk clotting enzymes in whey using the diffusion assay.
Whey utilization

From every 10 pounds of milk used for cheese making, approximately 9 pounds of liquid whey are obtained for each pound of cheese. Liquid whey after separation of fat contains about 6.3 percent total solids, consisting of 4.9 percent lactose, .9 percent protein and .5 percent ash (2, 14, 48).

As cheese production has increased, problems related to whey disposal have increased. Environmentalists have looked with alarm at increased whey production and its effect on pollution of rivers and streams. Humanitarians see whey as a potential source of food going down the drain. Researchers have tried to develop uses for whey and its constituents. Cheese manufacturers, whey processors and city officials have sought ways to dispose of whey with the least expense. Many people have tried to convert whey to an economically profitable commodity (36).

It was estimated that in 1973, 30.5 billion pounds of liquid whey were produced with only 43 percent processed (95). The excess was diverted into some type of sewage system (2, 51, 53, 73). Whey disposed of in this manner becomes a pollutant in rivers and streams in the area of disposal. The Water Quality Act of 1965 provided for water quality standards for the first time in Federal legislation. This act was followed by the Clean Water Restoration Act in 1966. These two acts provided for sewage plant construction plus research and demonstration.
programs for water pollution control. As a result of the 1966 Act the Federal Water Quality Administration established research and development programs specifically related to the dairy industry (54). The addition of whey to streams or sewage treatment plants reduces the oxygen content of the water; the oxygen being required to sustain aquatic life and reduce harmful bacteria (2, 53). Methods that have been proposed to reduce pollution with whey include use of whey in a liquid form for animal consumption, condensation or drying of the whey to recover the solids or fractions of the solids for incorporation into food products or animal feeds, and disposal of the liquid whey on the land in a manner that would not create pollution problems. With an increase in transportation distances and the large volumes produced, liquid whey has had limited success as an animal feed (2, 33). Swine have traditionally been the major animal consumers of liquid whey. Studies have been performed on the utilization of liquid whey by dairy animals (33). It was found that the consumption of liquid whey was dependent on the feed available to the animals, more whey being consumed by animals fed dry rations. Farmers were reluctant to use much whey because of the added inconvenience, equipment requirements and cost of purchasing the liquid whey. Concentration of whey by various means to obtain the solids or fractions of these solids has been the method of utilization of most interest due to potential monetary return. Use of whey as a fertilizer has both potential and built in limitations. Whey contains many plant nutrients but it can still become a pollutant if not completely absorbed (2).
A great deal of research activity has been directed toward processing liquid whey to obtain dry or concentrated liquid products for incorporation into food for man or animals.

Vaughan (94) proposed whey as a dietary supplement for populations of the world that were eating low quality foods. He compared the dietary value of nonfat-dry milk with that of dried whey and whey concentrated by reverse osmosis. He found that the protein efficiency ratios of rat diets supplemented with dried whey were lower than those supplemented with nonfat-dry milk. Those diets supplemented with whey concentrated by reverse osmosis had higher protein efficiency ratios, than those supplemented with nonfat-dry milk.

Protein efficiency ratios were calculated on the basis of grams of weight gained per gram of protein eaten. This preliminary study on the nutritional aspects will naturally need further analysis because of the differences in types of protein present in nonfat-dry milk and whey. Also the study was performed on rats and the true test as to whether these findings are applicable to humans will have to be tested on humans. Lactose intolerance will be one problem of concern because the lactose in dried whey is higher than the lactose in nonfat-dry milk (94).

Whey has been processed by various means to concentrate the solids for further use in many products.

One method that has been used to process whey is drying. Dry cheese whey is the second most important dehydrated milk by product in terms of volume (76). Sweet whey has been dried in the same manner as nonfat-dry milk. The steps involved in drying whey have been reviewed by Young (102) and Pallansch (75). Whey drying usually involves
(a) preheating, (b) concentration, (c) precrystalization, (d) spray drying, (e) after-crystalization, (f) fluid-bed drying, (g) pneumatic cooling, and (h) fluid-bed cooling. The number of steps varying with the specific unit involved. They suggested the following procedures be used prior to spray drying. The unconcentrated whey was preheated to 150-165°F and held at that temperature for the period of time dictated by the drying method. Agitation was necessary to avoid sludge formation. The whey was then heated to 195-205°F and held at that temperature until entering the evaporator for concentration. The first effect temperatures of the evaporator ranged from 170-195°F with 125 to 135°F second effect temperatures. The recommended total solids levels of the concentrated whey ranged from 40 to 50 percent or higher, based on the drying method employed. The concentrate from the evaporator was either dried immediately after evaporation or stored to be processed later.

A highly hygroscopic dried whey resulted when a one stage method was used upon drying the concentrate directly from the evaporator. Non-hygroscopic powder was produced by a two-stage method which allowed crystalization of the lactose prior to final drying to a level below 4 percent moisture. Another technique used to produce non-hygroscopic whey powder was the cooling of the concentrated whey, storage and agitation at the cooled temperature which allowed crystalization of the lactose prior to drying. Lactose crystals were used to seed the concentrated whey.

Concentration by the use of a vacuum pan has been used to concentrate liquid whey (98). Due to the highly perishable nature of whey, condensation in a vacuum pan to reduce the water content improves the keeping
quality and lets the small producer transport less water and more solids to the whey processor. Condensed whey has been made by pasteurizing the whey and drawing the hot liquid directly into the vacuum pan where the water was boiled off under a vacuum at low temperatures. Whey has been condensed to a total solids level up to 70 percent. Webb (98) also reports use of sweetened condensed whey in products where sugar was to be added.

Another method for concentrating whey has been proposed for use in the dairy industry. The process of reverse osmosis, a process for separating the components of a solution using a semi-permeable membrane, has been used for the concentration of whey (30, 66, 67, 68). The semi-permeable membrane allows the solvent (water) to pass through while impeding the passage of solutes in the water. Thus water was forced to pass through the membrane in a direction opposite the flow of osmosis. Hydrostatic pressure in excess of osmotic pressure was used as the driving force. McDonough (67) proposed reverse osmosis as an economical means for the small cheese manufacturer to concentrate his whey and dispose of it with lower cost and potentially higher profit than drying and condensing methods.

Pilot plant operations have been studied for the concentration of whey solids (68, 103). A 4 to 1 concentration level was found to be most practical, with higher levels producing fouled membranes (68). Pressures used to induce the reverse osmosis were reported from 600 to 800 pounds per square inch. The pressure requirement increased as concentration proceeded.
Problems encountered in the pilot plant tests were: reduced permeability of the membrane due to fouling by the solutes, compaction of the membrane after long periods of use, and high bacterial loads if the system was shut down for three to five days (68, 103). The fouling problem was partially resolved by creating turbulent flow within the system. Improved membranes have curbed the compaction problem. Cleaning the system with specially chosen sanitizing agents has reduced contamination.

Ultrafiltration has also been proposed as a means of whey processing. Both reverse osmosis and ultrafiltration use membranes for their specific action. Reverse osmosis removes only the water, where ultrafiltration is selective in which constituents of a solution are allowed to pass through the membrane. The pore size of the membrane was determined according to the solute to be removed. The pressures used in ultrafiltration were much lower than those of a reverse osmosis system. Fifty pounds per square inch gage pressure was reported as a maximum by Zall and Goldstein (103). Fenton-May (30) suggested ultrafiltration as a method of concentrating whey solids prior to evaporation.

Ultrafiltration and reverse osmosis have been used in combination for the concentration and fractionation of whey solids (68, 103).

Concentrated whey solids (dry or liquid concentrate) have been used in many food products. Animal feeds (33, 47, 65), bakery goods (37, 47), beverages (20, 38, 50, 51, 70, 73, 101), sausage products (49), confections (1), and dairy products (20, 44, 51, 90, 99) have had whey added as a constituent. Several reviews of whey utilization are available (47, 51, 78, 79).
**Rennet substitutes**

Rennin, a calf gastric enzyme in the form of a crude extract called rennet, has had almost exclusive use in the cheese industry for the setting of cheese milk. In recent years, due to the rapid growth of the cheese industry and the shortage of rennet, rennet substitutes have been found and developed for commercial use by the cheese maker. Nearly all proteolytic enzymes are capable of clotting milk. Thus, milk clotting enzymes have been obtained from every class of living organisms (23). Bacterial enzymes have been isolated and evaluated (18, 23, 46, 61). Clotting enzymes from higher plants have been reported (23, 52). Fungal protease preparations have found acceptance as milk clotting enzymes (3, 4, 5, 16, 23, 61, 71, 72, 74, 87, 92, 93). Several animal proteases have been investigated. Only pepsin (porcine and bovine) and rennin have interested the cheese industry (18, 22, 23, 24, 31, 35, 58, 59, 60, 62, 63, 69, 72, 84).

A microbial-derived rennet substitute would be of value due to the limitless supply potential. Thousands of bacteria have been studied in the quest for a suitable rennet substitute. Very few bacterial enzymes have been used for cheese making. Cheeses made with these bacteria proteases have been criticized for off-flavors and poor body and texture due to excessive proteolysis (61, 69, 80, 81, 100). Bacterial proteases, therefore, are not used commercially for manufacture of cheese.

Plant proteases have not been successful as rennet substitutes (23, 52). These enzymes are strongly proteolytic and cause bitter flavors, pasty body and reduced yields.
Lucas (58) and Merker (62) in the early 1900's suggested the use of swine pepsin for cheese making due to the shortage of calf vells for rennet production. Substantial use of pepsin as a rennet substitute was not evidenced until the 1960's when production of cheese increased to the point that rennet producers could not provide sufficient rennet extract. Shortages of calf vell supplies also added to this situation. Since then many researchers have recommended pepsin as a satisfactory substitute for all or part of the rennet used to make Cheddar cheese (24, 31, 35, 49, 60).

Due to the unstable nature of porcine pepsin above pH 6.5, the National Cheese Institute (69) in 1960 recommended that a mixture of rennet and porcine pepsin be used for manufacture of Cheddar cheese. Slow coagulation of milk resulted from this instability, and a soft curd formed which resulted in high fat losses upon cutting the curd. Ernstrom (24) found that the clotting time of skim milk treated with pepsin increased rapidly between pH 6.5 and 6.7 and at pH 6.8 normal levels of pepsin failed to clot skim milk. Emmons (22) suggested inactivation of pepsin when diluted with hard water at high pH. Above pH 5.5 pepsin is pH dependent. The pH range of special significance is from 6.0 to 6.7 where the greatest inactivation occurs. Green (35) suggested that during Cheddar cheese manufacture most of the porcine pepsin activity was destroyed. Wang (96) was unable to detect residual pepsin activity in fresh Cheddar cheese curd.

Linklater (57) showed that commercial rennet extracts contain small amounts of bovine pepsin. The maximum level he reported was 3.7 percent of the clotting activity due to the pepsin. Green (35) made comparable
quality cheddar cheese from milk set with rennet and bovine pepsin. Fox (31) noted only slight activity variations between rennet and bovine pepsin. Bovine pepsin has not been used as such for cheese manufacture but generally has been classified as a contaminent in rennet extractions (57). Depending upon further research and legislative clarification bovine pepsin could be used as a rennet substitute (23).

The most successful rennet substitutes have been proteolytic enzymes of fungal origin. Proteases from Endothia parasitica, Mucor pusillus var. Lindt and Mucor miehei have been considered safe and suitable rennet substitutes (27, 28, 29).

Cheddar cheese made (87) by utilizing a preparation of Endothia parasitica protease (EP protease) had a bitter flavor and poor body when compared to a control made with rennet. EP protease was more proteolytic than rennet (64, 93, 100). When used in the manufacture of cheeses made with high cooking temperatures bitterness did not occur and the resulting cheeses were of excellent quality. Whitaker (100) and Sardinas (87) noted that EP protease was sensitive to high heat. It has been postulated that Endothia parasitica protease was inactivated by the high cooking temperatures and therefore did not affect the cheese during curing.

Another successful fungal rennet substitute has been derived from Mucor pusillus var. Lindt. Mucor pusillus var. Lindt protease (MP protease) has been used to make many types of cheese and is now being used commercially (4, 5, 71, 85, 86). Arima and Iwasaki (3, 4) patented methods for preparation of the enzyme and a technique for cheese making using MP protease. Richardson et al (85) found only slight bitterness
in Cheddar cheese made with MP protease after 14 months curing. MP protease was less proteolytic than EP protease but both were more proteolytic than rennin (64, 93).

A protease from Mucor miehei (MM protease) has also proved useful in cheese manufacture. Christensen (16) reported the use of MM protease for the manufacture of 15 varieties of cheese in the United States, France and Germany. It was more proteolytic than rennin and had a wide pH range of stability. Ottesen and Rickert (74) presented data showing retention of 90 percent of the activity after an eight day heat treatment at 38 C at pH values between 3.0 and 6.0. Bitterness has not been reported in cheeses made with MM protease. Sternberg (92) reported that experimental cheese made with calf rennet and MM protease were indistinguishable.

Measuring enzyme activity

Enzyme activity is usually determined by measuring the rate at which products of the enzyme-catalyzed reaction appear, or the rate of substrate decomposition. Milk clotting is a complex process with both a primary enzymatic phase in which \( k \)-casein is altered and its stabilizing effect on the remainder of the caseinate complex is altered, and a secondary phase, a non-enzymatic process, in which aggregation of the altered caseinate takes place (23). Hill (40) and Hill et al (41) noted that simple peptides containing the phenylalanine-methionine bond, that is known to be hydrolyzed in \( k \)-casein, was not readily hydrolyzed at pH values where rennin clots milk. Thus, the object of a great deal of research has been the determination of conditions necessary for the specific enzyme attack on \( k \)-casein (6, 9, 45, 55, 97). Also, the
development of a simple substrate which could be used to follow the primary action of milk clotting enzymes has been investigated (23).

The secondary phase of milk clotting is susceptible to variation in milk composition. Thus, differences in clotting times due to natural variations in milk composition, have made it difficult to develop a standard milk substrate for enzyme assays.

Berridge (11) described a substrate made by reconstituting 12g nonfat dry milk in 100 ml of 0.01 M CaCl₂. Ten milliliters of this substrate were tempered in test tubes at 30 C in a water bath for 30 minutes and then inoculated with one milliliter of diluted rennet extract. The end point was taken as the first visible formation of flakes in a flowing film of milk. A stirring rod was dipped into the substrate and touched to the side of the test tube, this allowed the milk adhering to it to flow down the side of the test tube, thus forming a flowing film. This procedure was repeated until the end point was observed.

Bakker, Scheffers and Wiken (7) altered the Berridge method by placing a small test tube inside the tube used by Berridge thus forming a film without the need to dip a rod into the substrate. The sensitivity was the same.

An apparatus to test the clotting time of mastitic milk was described by Sommer and Matsen (91). One hundred and twenty five milliliter wide-mouth bottles were rotated in an inclined position in a water bath, this provided a continuous film of milk on the inner surface of the bottle. Fifty milliliters of the milk tempered to 30 C were inoculated with one milliliter of a 1:50 dilution of rennet extract. Timers attached to the apparatus were used to measure the clotting time, with
the end point being indicated by flake formation in the film of milk. Berridge (10) also used a method for automatic formation of a flowing milk film on the inner surface of boiling tubes by rotating them in an inclined position in a water bath. Ernstrom (25) combined the Berridge substrate and the Sommer-Matsen apparatus to measure rennin activities. He suggested aging the substrate for 20 hours at 2°C before use. He had previously found that the clotting time continued to increase for 20 hours after preparation when the Berridge substrate was stored at 2°C. The activity in a rennet extract of unknown strength was measured by the time required for one milliliter of an appropriate dilution of the unknown extract to clot 25 ml of Berridge substrate. This was compared to the time required for one milliliter of a known dilution of a standard strength rennet extract to clot 25 ml of an identical substrate run at the same time in the tester. The strength of the undiluted standard was arbitrarily assigned the value of 100 rennin units (RU) per milliliter. The activity in the unknown was related to the standard by the equation:

\[ \text{RU/ml} = 100 \frac{\text{Ts}}{\text{Tu}} \times \frac{\text{Cs}}{\text{Cu}} \]

where
- \( \text{RU} \) = rennin units
- \( \text{Ts} \) = coagulation time of standard
- \( \text{Tu} \) = coagulation time of unknown
- \( \text{Cu} \) = concentration of unknown
- \( \text{Cs} \) = concentration of standard
- 100 = rennin units in 1 ml of undiluted standard
The comparisons made were performed by diluting the enzymes to a concentration that would give approximately the same clotting time as the standard.

The strength of the standard rennet extracts over a period of years is difficult to assess, this fact points out the drawbacks of this method. Renewal of standard solutions is necessary to maintain uniformity of the assay.

New batches of nonfat dry milk must be checked against the old with an enzyme solution of known activity due to variations in clotting times of different samples of reconstituted nonfat dry milk. Introduction of new non-reennin coagulants have compounded the problems of standardizing the milk clotting activity of enzyme solutions. Mechanical limitations also become apparent in that only a few samples can be run at one time. These problems in standardizing rennin activity make it difficult for research workers in different laboratories to compare their results. A combination of the Sommer-Matson apparatus and Berridge substrate is the most common rennin activity test accepted and used by rennet control laboratories.

Other methods for assaying enzyme have been proposed. DeMan and Batra (19) adapted an automatic blood-clot timer for measuring rennet-clotting activity in milk. The test was fast, used less substrate and detected the end point automatically.

Due to the high ratio of enzyme volume to substrate volume precautions had to be taken to prevent differences in pH or salt concentration in enzyme solutions from affecting the clotting time.
Changes in optical density were used to continuously follow rennet coagulation by Claesson and Nitschmann (17). The graphs of light intensity versus time showed the beginning and progress of aggregation, formation of the gel and alterations inside the gel during aging. It was not possible to formulate the kinetics of the coagulation process by this method and find a velocity constant which was truly constant and independent of time and concentration.

Scott-Blair and Burnett (88) described a simple reproducible method for detecting the rennet-clotting time of milk based on changes in viscosiy. They found that by plotting viscosiy changes due to the action of rennet on the casein versus time that the same slope was always produced. Variance in slope was noted for different rennet extracts. This was attributed to protelytic enzymes other than rennin present in the different rennet extracts.

Everson and Winder (26) adapted an instrument designed to detect sound velocity in liquids to determine the coagulation time of renneted milk. At coagulation, a large increase in sound velocity occurred and was recorded automatically as the end point of the test. This method gave precise, and reproducible results with a recorded end point.

Low concentrations of rennin present in curd and whey are below the sensitivity of the afore mentioned tests including the combination of the Sommer-Matsen apparatus and Berridge substrate method. Reyes (82) used a procedure which involved the use of the very sensitive substrate devised by Gorini and Lanzavechia (34) and modified by Wang (96) for measuring residual rennin in curd and whey.
This method allowed determination of levels of rennin activity as low as .01 RU/ml. It was approximately 12 times more sensitive than the Berridge substrate. The substrate was so sensitive that the salts and small amounts of casein present with the rennin in the whey samples to be tested would alter the coagulation time. In order to obtain accurate results it was necessary to dilute the standard enzyme solution with heat treated whey. Since heat treatments affect the solubility of salts and also denature serum proteins in whey, it was impossible to know whether the heating of the whey altered the effect on coagulation of the substrate. The substrate was relatively unstable due to the sensitivity, making great care mandatory to ensure standard conditions during the test.

Elliot and Emmons (21) utilized a passive indirect hemagglutination test and a corresponding inhibition test to determine specific enzyme preparations. They also noted that levels of enzyme as low as a few ug/g of cheese could be detected. This assay was not quantitative as it was used but potentially could be made so.

Radial diffusion in casein-agar gels has been used by several investigators (15, 18, 32, 56, 93) to differentiate and characterize milk coagulants and mixtures of two or more enzymes. Due to the high concentration of enzyme tested the results were not quantitative.

Cheeseman (15) attempted to make an agar diffusion assay. The agar gel contained 1 percent whole casein, 10-20 mM-calcium chloride, 1 percent sodium acetate, and 1 percent agar with the pH being adjusted to 5.7 and 6.1. This substrate was placed in agar assay plates, cooled and had wells cut into the agar into which undiluted commercial rennet
was placed. The enzyme was allowed to diffuse for 48 hours at room temperature. He noted that two precipitation zones appeared in the form of rings separated by a clear zone when rennet was used. An attempt was made to determine concentration by diluting the rennet up to 1/1000 but the results were stated to be less sensitive than the Berridge (11) technique for milk clotting activity. This was probably due to the high concentrations of enzyme used. Cheeseman (15) did note that as different enzymes diffused through the agar there was a distinct difference in the precipitation zones produced. Also if casein fractions were used precipitation zones were altered.

Ganguli and Bhalerao (32) used the Cheeseman procedure (15) to demonstrate varying activities of animal, vegetable and microbial enzymes on buffalo and cow casein. Due to differences in precipitation zone patterns and numbers, they concluded that each enzyme had a different mode of action on the caseins therefore they stated that this technique could be used as a means of differentiating enzymes.

De Koning, et al (18) used starch-gel amylase assays and paper electrophoresis, along with the casein-agar diffusion technique to characterize milk coagulating enzymes. They suggested use of this assay only for unmixed enzymes when trying to characterize the enzymes. They too noted the differentiation in precipitation zone formation by each enzyme used.

Richardson (83) used casein-agar gels adjusted to pH 6.85 in 90 mm petri dishes to differentiate milk clotting enzymes. Twelve millimeter holes were bored into the solidified gel and 0.2 ml of enzyme solution was placed in the well formed. The inoculated casein-agar gels were
incubated at 32°C and checked every 24 hours. The work reported showed definite differences in precipitation patterns produced by rennet, pepsin, MP protease and EP protease. Variations in the secondary precipitation zone for commercial rennet extracts used by Richardson (83) could be due to the potential presence of differing levels of bovine pepsin in the commercial extracts as noted by Linklater (57). Richardson also assayed mixtures of fungal enzymes and rennet. This combination, upon assay showed the primary heavy zone formed by rennet followed by a much sharper heavy fungal zone. This work also pointed out that the technique as followed was still less sensitive than the standard milk clotting test of Berridge (11).

Lawrence and Sanderson (56) modified the Cheeseman procedure by using a thin layer of casein-agar on a defined area of a microscope slide. The rate of the radial diffusion of the enzyme in the casein-agar substrate was a measure of enzyme concentration. The number and width of the precipitation zones formed was dependant upon the enzyme used, the concentration of calcium ion, pH and temperature. As mentioned previously the different enzymes have been differentiated by their action on the casein as they diffuse through the agar (15, 18, 32, 56). Addition of calcium increased the sensitivity of the assay. Distinct white zones were obtained only if the pH was between 5.0 and 6.5. Temperature caused a variation in the number of bands formed. The sensitivity of the Cheeseman (15) substrate was improved by the use of thin casein-agar gels and reduction of the casein from 1 percent to 0.5 percent in the substrate.
The Lawrence and Sanderson technique (56) was sensitive and reproducible to the point that concentrations of 0.1 ug/ml of milk clotting enzyme could be quantitated. Drawbacks of this method include the use of special equipment for humidity control to prevent drying of the gels during incubation and the necessity of uniform thin layers of casein-agar on the microscope slide.

Methods for the detection and quantitation of enterotoxins in food include gel diffusion techniques (8, 12, 39). The concentration of enterotoxin was calculated by referring the diffusion distance to a standard curve prepared from known concentrations of enterotoxin plotted against diffusion distances. The diffusion distance was indicated by a precipitation band at the antigen-antibody interface. The antigen solution is layered over an immune-serum agar substrate placed in a thin bore glass tube. As more antigen diffuses into the agar, an antigen-antibody precipitate forms which progressively moves down the tube. There was a straight line relationship between the distance traveled by the leading edge of the band and the square root of time (8). This follows Fick's Law (13) \[ \frac{dc}{dt} = D \frac{\partial^2 c}{\partial x^2} \] where \( c \) = concentration, \( D \) = diffusion coefficient, \( x \) = distance diffused, and \( t \) = time.

Thus yielding an expression for solute concentration as a function of time and distance moved. The linear relationship means that the rate of diffusion of the leading edge of the precipitation band is related to some fixed concentration of antigen as it diffuses down the tube. The density of the precipitate at the leading edge of the band is a function of the antibody concentration only and independent of the concentration of antigen (8).
Using the principles of gel diffusion suggested by this technique plus a substrate similar to that used by Lawrence and Sanderson (56), Holmes (42) was able to develop a method for the estimation of low concentrations of milk clotting enzymes. He used a casein-agar substrate placed in 3 mm x 110 mm sedimentation tubes. The substrate contained .5 percent casein, 3.6 percent sodium acetate, .01 percent CaCl₂, and .7 percent Ion Agar. A five microliter sample of the enzyme solution being tested was placed on the surface of the casein-agar substrate and allowed to diffuse through the casein-agar. A white precipitation band formed by the action of the enzyme on the caseinate marked the progress of the enzyme as it diffused through the substrate. The distance diffused in a given time was proportional to the enzyme concentration. It was reported that the density of the precipitate at the leading edge of the band was a function of casein concentration and was independent of enzyme concentration. Concentrations as low as $1 \times 10^{-4}$ RU/ml with a 5 percent standard deviation were detectable with lower concentrations undetected or deviating from the straight line relationship.

When concentrations of $10^{-1}$ RU/ml or higher (42) were tested, the conventional clotting test with Berridge substrate was more precise and convenient. The diffusion followed Fick's law in that the relationship between diffusion distance and the square root of time was linear. Holmes was able to determine the distribution of milk clotting enzymes between Cheddar cheese curd and whey using this assay. The distribution of rennet between curd and whey was affected by the pH of the milk at setting, the higher the pH the more enzyme released to
the whey. Porcine pepsin distribution was also pH dependant. *Mucor pusillus var. Lindt* protease was not affected by pH. Holmes was able to account for $102 \pm 5$ percent of the rennet and $99 \pm 2$ percent of the *Mucor pusillus* protease added to the cheese milk after testing enzyme levels in the curd and whey.

Advantages over previous methods of enzyme analysis were listed: greater sensitivity, numerous tests run simultaneously, preparation of diffusion tubes in advance, diffusion distances easily measured with a densitometer, and no effect of whey solids and salt on diffusion. Disadvantages were 48 hour incubation time before measuring, a requirement for high quality casein in the substrate and clouding or partial precipitation of the casein in the substrate. Diffusion distances were often masked by this clouding and diffusion bands frequently could not be detected.

Resolution of these disadvantages is an area in which further research should be performed. With the ability to detect low levels of milk clotting enzymes many theories proposed by early researchers can be refuted or substantiated and improved cheese and whey products can be developed.

It may be possible to follow the survival of milk clotting enzymes left in cheese curd during the cheese ripening process.
MATERIALS AND METHODS

Milk

Raw whole milk for cheese making was obtained from Utah State University Dairy Farm. The milk was pasteurized by 63°C for 30 minutes, cooled and stored at 2°C overnight.

Enzymes

Enzymes used in this study included rennet extracted from New Zealand bobby calf vells\textsuperscript{1}, porcine pepsin\textsuperscript{2}, bovine pepsin\textsuperscript{3}, proteases derived from \textit{Mucor pusillus var. Lindt} (MP protease)\textsuperscript{4}, \textit{Mucor miehei} (MM protease)\textsuperscript{5}, and \textit{Endothia parasitica} (EP protease)\textsuperscript{2}, and a standard rennet extract\textsuperscript{4}. The standard rennet extract was assigned a value of 100 rennin units (RU) of activity per milliliter, and the activities of the other enzymes were evaluated against the standard by the method of Ernstrom (25).

\textsuperscript{1}Obtained from New Zealand Cooperative Rennet Company, Eltham, New Zealand.

\textsuperscript{2}Obtained from Chas. Pfizer Inc., Pfizer Chemicals Division, New York, New York.

\textsuperscript{3}Obtained from Chas. Pfizer Inc., Pfizer Chemicals Division, New York, New York. Bovine pepsin was prepared by John Shovers of Pfizer Chemicals and was determined rennin-free by him.

\textsuperscript{4}Obtained from Dairyland Food Laboratories, Waukesha, Wisconsin.

\textsuperscript{5}Obtained from Miles Laboratories Inc., Marshall Division, Madison, Wisconsin.
Preparation of kappa-casein

The method of Zittle and Custer (104) was followed for the preparation of kappa-casein. Low lipid level allowed deletion of the ethanol extraction. Purified casein was also used as a replacement for the frozen block of acid precipitated whole casein recommended by Zittle and Custer (104) (100 grams of casein were used).

Preparation of the kappa-casein-agar substrate

The substrate used by Holmes (42) was modified by replacing the whole casein with kappa-casein, deleting the CaCl₂, raising the pH to 5.9 and using Bacto-Agar in place of Ion Agar. The substrate contained .54 percent kappa-casein, 3.6 percent sodium acetate, and .73 percent Bacto-Agar.

1. Six-tenths grams of kappa-casein and .6 g NaC₂H₃O₂·3H₂O were weighed into a prepared 100 ml beaker. Distilled water was added to bring the weight to 18 g. The solution was stirred with a magnetic stirrer until the kappa-casein was dissolved (10-30 min). The pH was carefully adjusted to 5.9 with .1N HCL. Distilled water was added to bring the weight to 20 g. This solution was identified as the "kappa-casein solution."

2. Six grams of NaC₃H₆O₂·3H₂O and .8 g of Bacto-Agar were weighed in a prepared 125 ml erlenmeyer flask and 80 ml of distilled water was added. The pH was adjusted to 5.9 with LN HCL and the solution was

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¹Purified casein used was prepared by Fisher Scientific Company, Chemical Manufacturing Division, Fair Lawn, New Jersey.

²Bacto-Agar #0140, Difco Laboratories (used because it was less expensive than Ion Agar and was easier to locate).
brought to a weight of 90 g with distilled water. The solution was autoclaved for 10 minutes at 15 pounds pressure and cooled to 75 C. The solution was identified as the "agar solution."

3. The kappa-casein and agar solutions were brought to approximately 75 C in a water bath and mixed. This mixture was known as the "kappa-casein-agar solution."

**Preparation of the diffusion tubes**

The procedure recommended by Holmes (42) was followed. Sedimentation tubes 1 (3mm ID x 110 mm length) were three-fourths filled with hot kappa-casein agar solution with a 10 ml syringe fitted with a plastic tube. The tubes were sealed with paraffin-candy wax mixture and stored at 2 C until used.

**Measuring enzyme concentration**

The procedure outlined by Holmes (42) was followed. The diffusion tubes were removed from the refrigerator and allowed to come to room temperature. The wax seals were removed and 5 µl of the sample containing the enzyme was placed on the surface of the kappa-casein-agar gel. Sampling was done with a 10 µl syringe. Enzyme solutions of known concentration were assayed at the same time as the unknown samples. The tubes were resealed with wax and placed in racks with holes drilled to hold the diffusion tubes in an upright position for 48 hours at 37 C. The diffusion distance was indicated by a white precipitation band

1Tubes, Becton-Dickson disposable sedimentation and hematocrit tube, 5 x 115 mm, #6901.
caused by the action of the enzyme on the caseinate. Diffusion distances were determined by measuring the distance of the leading edge of the precipitation band from the origin. Distances were measured with a transmission densitometer\(^1\) and recorded on a Heathkit millivolt recorder\(^2\).

A special plastic tray was made to fit the template frame of the densitometer. This tray held the diffusion tubes parallel to the sensor head. Spacing between grooves was 1 cm to allow for ease in placing and removing the diffusion tubes.

The recorder tracings were measured as shown in Figure 1. A standard curve was prepared by plotting the diffusion distance versus enzyme concentration on semilogarithmic graph paper. From this standard curve the concentration of the unknown enzyme sample was determined.

**Cheddar cheese making**

Cheddar cheese was made by a modification of the Price and Calbert method (77). Sixteen pounds (7.26 kg) of pasteurized milk were placed into 20 cm cubical plastic vats. One percent starter was added at 31 C and the milk was set with the equivalent of 85 g of enzyme preparation per 454 kg of milk. The cheese was cooked to 39 C (102 F) and held at that temperature for 60 min. At this point the whey was drained. Whey samples were taken upon reaching a cooking temperature of 39 C.

\(^1\) Densitometer, \#C 910 transmission densitometer, EC Apparatus Corp., 5000 Bark Street N., St. Petersburgs, Florida 33733.

\(^2\) Millivolt recorder, Heath Servo-recorder, model EU-20B, Heath Company, Benton Harber, Michigan 49022.
Figure 1. Typical densitometer-recorder sketchings of enzyme diffusion in whole casein-agar and kappa-casein-agar.
and at draining 60 minutes later. The curd was discarded. Heating was accomplished by electrical resistance (42).

**Heat treatment of whey samples**

Samples of whey were adjusted to pH 5.2, 5.6, 6.0, 6.2, 6.6, 7.0 with 1N HCL or 1N NaOH. They were then placed in 11 mm x 100 mm test tubes and subjected to the following heat treatments: 68.3 C, 71.1 C and 73.9 C for 0.25, 0.5, 1, 5, and 10 minutes. Heating was accomplished by placing the tubes in a water bath set at 87.8 C and agitating them vigorously until the desired temperature was reached. At which point the tubes were placed in a water bath set at the desired temperature. The tubes were placed directly into an ice water bath after being held for the specified time. Due to the size of the test tubes, a recording thermocouple¹ was used to determine the temperature.

**Measurement of enzyme concentration in whey**

Whey samples were assayed before and after heat treatment by placing 5 ul of undiluted whey on the surface of the kappa-casein-agar gel. Incubation, measurement of diffusion distance, and determination of concentration were performed as previously explained. Results were expressed as a percentage of original activity of the unheated whey.

RESULTS

Effect of replacing whole casein with kappa-casein in the test substrate

Holmes (42) reported that clouding would sometimes occur in the casein-agar diffusion gels that he used for enzyme assay. Elimination of this clouding was one objective of this project. Kappa-casein is readily acted upon by milk clotting enzymes (6, 23, 45, 55, 64, 97, 105). Kappa-casein was used to replace the whole casein in the Holmes substrate. This replacement provided much clearer tubes that were not susceptible to clouding. There was no need for adding CaCl₂ and a less expensive and more available agar could be used. Figure 2 is a photograph comparing a clouded whole casein-agar gel and a clear kappa-casein-agar gel. The clarity of the substrate resulted in sharper diffusion boundaries. Figure 1 shows typical densitometer-recorder sketchings of enzyme diffusion in tubes made with whole casein and kappa-casein. The kappa-casein sketch shows a rapid drop as the densitometer nears the leading edge of the diffusion band. The measurement of the diffusion distance was slightly different than the method used by Holmes. The leading edge of the diffusion band was marked as the point at which a sudden deviation was noticed in the downward path of the peak as recorded by the densitometer-recorder. Due to the cloudiness of the whole casein-agar, Holmes (42) determined the leading edge of the diffusion band as the point where the peak started the downward path.
Figure 2. Whole casein-agar and kappa-casein-agar diffusion tubes. (Note the clouding in the whole casein-agar tube and the clarity of the kappa-casein-agar tube.)
Effect of cooking time on residual enzyme activity

The protease activity of the six milk clotting enzymes in whey tested before and after cheese cooking at 39 C is listed in Table 1.

Table 1. Protease activity of six milk clotting enzymes before and after heating to 39 C for 60 min.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Cooking time at 39 C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 minutes</td>
</tr>
<tr>
<td></td>
<td>(RU/ml)</td>
</tr>
<tr>
<td>Rennet</td>
<td>2.1 x 10^-2</td>
</tr>
<tr>
<td>Bovine pepsin</td>
<td>2.2 x 10^-2</td>
</tr>
<tr>
<td>Porcine pepsin</td>
<td>1.9 x 10^-2</td>
</tr>
<tr>
<td>MM protease</td>
<td>2.0 x 10^-2</td>
</tr>
<tr>
<td>MP protease</td>
<td>2.2 x 10^-2</td>
</tr>
<tr>
<td>EP protease</td>
<td>2.1 x 10^-2</td>
</tr>
</tbody>
</table>

Effect of heat on enzyme stability in whey

*Mucor miehei* protease. Figures 3 through 5 show the percent of original *Mucor miehei* protease left in cheese whey after exposure to 68.3 C, 71.1 C and 73.9 C for one quarter to ten minutes at six different pH values. At 68.3 C (Figure 3) and at pH 5.2, approximately 75 percent of the original MM protease activity remained in the whey after a 10 minute heat treatment. As the pH increased the heat stability of the enzyme decreased. The heat stability dropped faster at 71.1 C (Figure 4). Residual enzyme activity was still detectable at pH 6.2-5.2
Figure 3. Percent of original *Mucor miehei* protease activity left in cheese whey after exposure to 68.3°C for one quarter to 10 minutes at six different pH values.

*No activity was found in samples heated at these pH values.*
Figure 4. Percent of original *Mucor miehei* protease activity left in cheese whey after exposure to 71.1°C for one quarter to 10 minutes at six different pH values.

+No activity was found in samples heated at these pH values.
Figure 5. Percent of original Mucor miehei protease activity left in cheese whey after exposure to 73.9 °C for one quarter to 10 minutes at six different pH values.

+No activity was found in samples heated at these pH values.
after 10 minutes at 71.1 C. Greater stability was noted in samples heated at lower pH values. Figure 5 shows an even faster decline in stability of MM protease heated at 73.9 C. Again as the pH increased the heat stability decreased. There was still residual activity in samples adjusted to pH 5.2-6.0 after a 10 minute heat treatment. No sample adjusted to pH 7.0 survived the minimum heat treatment for 15 seconds.

*Mucor pusillus* var. *Lindt protease*. The percent of original MP protease activity left in cheese whey after exposure to 68.3 C, 71.1 C and 73.9 C for one quarter to 10 minutes at six different pH values is shown in Figures 6 through 8. The residual activity after heating at 68.3 C (Figure 6) dropped sharply. An increase in pH reduced the heat stability of MP protease as was noted with MM protease. Figures 7 and 8 show an even greater decline in heat stability of residual MP protease in whey after exposure to 71.1 C and 73.9 C.

*Rennet*. Residual rennet activity dropped sharply after heating at 68.3 C, 71.1 C and 73.9 C (Figures 9 through 11). Even after 15 seconds at 68.3 C only 20 percent of the original activity remained in the whey samples adjusted to pH 5.2. A decrease in pH caused an increase in heat stability of the enzyme.

*Bovine pepsin*. Bovine pepsin (Figures 12 and 13) was more heat sensitive than the previously mentioned enzymes. The residual activity dropped sharply at 68.3 C (Figure 12) and as the temperature was increased the residual activity dropped to a level that no residual activity was noted in samples heated at 73.9 C.
Figure 6. Percent of original *Mucor pusillus* var. *Lindt* protease activity left in cheese whey after exposure to 68.3°C for one quarter to 10 minutes at six different pH values.

+No activity was found in samples heated at these pH values.
Figure 7. Percent of original *Mucor pusillus* var. *Lindt* protease activity left in cheese whey after exposure to 71.1°C for one quarter to 10 minutes at six different pH values. +No activity was found in samples heated at these pH values.
Figure 8. Percent of original Mucor pusillus var. Lindt protease activity left in cheese whey after exposure to 73.9 C for one quarter to 10 minutes at six different pH values.

+No activity was found in samples heated at these pH values.
Figure 9. Percent of original rennet activity left in cheese whey after exposure to 68.3°C for one quarter to 10 minutes at six different pH values.

+No activity was found in samples heated at these pH values.
Figure 10. Percent of original rennet activity left in cheese whey after exposure to 71.1 °C for one quarter to 10 minutes at six different pH values.

+No activity was found in samples heated at these pH values.
Figure 11. Percent of original rennet activity left in cheese whey after exposure to 73.9 °C for one quarter to 10 minutes at six different pH values.

+No activity was found in samples heated at these pH values.
Figure 12. Percent of original bovine pepsin activity left in cheese whey after exposure to 68.3 °C for one quarter to 10 minutes at six different pH values.

+No activity was found in samples heated at these pH values.
Figure 13. Percent of original bovine pepsin activity left in cheese whey after exposure to 71.1°C for one quarter to 10 minutes at six different pH values.

+No activity was found in samples heated at these pH values.
**Endothia parasitica protease.** The percent of original EP protease activity left in cheese whey after exposure to 68.3°C for one quarter to 10 minutes at six different pH values is shown in Figure 14. Residual activity was reduced to below 5 percent after 15 seconds at 68.3°C. There was no survival of residual enzyme at the higher temperatures used in this study. Note that at pH 7.0 the enzyme was most stable in the other enzymes tested stability declined most rapidly at pH 7.0. Also with an increase in pH there was an increase in EP protease heat stability.

**Porcine pepsin.** Porcine pepsin was very heat sensitive at the pH values tested (Figure 15). Only samples adjusted to pH 5.2 showed any residual porcine pepsin activity when heated at 68.3°C.
Figure 14. Percent of original *Endothia parasitica* protease activity left in cheese whey after exposure to 68.3°C for one quarter to 10 minutes at six different pH values.

+No activity was found in samples heated at these pH values.
Figure 15. Percent of original porcine pepsin activity left in cheese whey after exposure to 68.3°C for one quarter to 10 minutes at six different pH values.

+No activity was found in samples heated at these pH values.
DISCUSSION

Clouding was eliminated in casein-agar gels used for the measurement of low concentrations of milk clotting enzymes by replacing whole casein with kappa-casein, eliminating the CaCl₂ and raising the pH to 5.9. Holmes (42) was frequently plagued with clouding of the diffusion tubes used in assaying milk clotting enzyme activity due to the instability of the casein at pH 5.7 and in the presence of CaCl₂. The use of kappa-casein to replace the whole casein not only eliminated clouding but also gave very clear diffusion tubes. Due to the clarity of the diffusion substrate the diffusion boundaries were sharpened. The density of the diffusion bands formed by the action of the enzymes on the kappa-casein was less than the density of bands formed in whole casein gels. This confirmed the work of Lawrence and Sanderson (56), who stated that diffusion bands in gels prepared with kappa-casein were lighter than bands formed when whole casein was used in the agar. This lighter band formation was a problem in their work because of the thin layer of gel used in their technique. Gels inside the diffusion tubes were much thicker than the thin layer gels used in the microscope slide method. The densitometer easily detected the diffusion band and the leading edge of the diffusion was marked by a rapid decrease in density due to the clarity of the substrate. Because the leading edge of the diffusion was so distinctly marked, the point of measuring the distance of the leading edge of the diffusion from the origin was changed as shown in Figure 1. When the first distinct change occurred in the downward slope
of the peak recorded by the densitometer, that point was called the leading edge.

Many new areas of research related to milk clotting enzymes can be performed using this assay technique. Ideas expressed by early investigators about the effect of milk clotting enzymes on cheese ripening can be investigated by analysis of the cheese during curing to determine the survival of the enzymes. Not only can research be done on dairy related areas but workers in other fields can use the principles of linear diffusion in specific substrates containing agars to detect the actions and concentration of enzymes in various systems.

The kappa-casein-agar gels made in this study allowed the evaluation of residual enzyme activity present in whey samples taken after a normal Cheddar cheese cook procedure of 39°C for 60 min. Samples of whey were assayed for activity upon reaching the cooking temperature and again 60 min later (Table 1). Porcine pepsin lost the most activity, almost 50 percent. Bovine pepsin followed with about a 14 percent loss. Both rennet and EP protease lost 5 percent and the two fungal enzymes MM protease and MP protease lost no activity. The use of higher cooking temperatures used in the manufacture of some varieties of cheese would probably inactivate more of the milk clotting enzymes.

Another area of question that was worked on in this study was the heat stability of these enzymes as they were treated in cheese whey. *Mucor miehei* protease was the most heat stable of the enzymes tested. Almost 75 percent of the original activity remained after a heat treatment of 68.3°C for 10 minutes in samples adjusted to pH 5.2 (Figure 3).

Figure 5 shows that detectable residual enzyme activity was found after a heat treatment of 73.9°C for 10 minutes in whey samples adjusted
to pH 5.2 and 5.6. With an increase in temperature the residual activity decreased; this was expected. *Mucor miehei* protease was the most heat stable at all pH values, followed by MP protease, rennet, bovine pepsin, EP protease and porcine pepsin.

Three conclusions that can be listed from this work are:

1. *Mucor miehei* protease was the most heat stable of all the milk clotting enzymes tested.

2. As the pH increased there was a decrease in the heat stability of the enzymes tested except for EP protease which reacted just the opposite (with an increase in pH there was an increase in heat stability).

3. At pH 7.0 the rate of inactivation of the enzymes occurred most readily except for EP protease which was most stable at that pH. At pH 5.2 the greatest stability was noted in the enzymes tested except EP protease which was inactivated most readily at that pH.

It should be noted that the stability of EP protease was affected only slightly by pH and the fact that it was most stable at pH 7.0 and reacted with an increase in stability as the pH increased was not as significant as it sounds because after 15 seconds at the minimum heat treatment the activity remaining was below 5 percent. This is probably the reason EP protease has found acceptance in the Swiss and Italian cheese industry. Both Swiss and Italian cheeses are made with high cooking temperatures. Cheddar cheese manufacturers do not use EP protease because a poor quality cheese is made due to the high proteolytic nature of the residual enzyme.
Using this technique, whey processors will be able to better determine the levels of residual milk clotting enzymes present in whey prior to and after processing. This knowledge would permit these processors to select the method of incorporating their product into the variety of milk containing food stuffs. Also a better understanding of the stability of the various milk clotting enzymes used in cheese manufacture will permit whey processors to regulate their processing temperatures, times and/or pH levels to insure inactivation of residual milk clotting enzymes in the whey.

This study suggests the potential of further studies on the stability of residual milk clotting enzymes. High temperature short time treatments on cheese whey should be evaluated. This is indicated by the fact that the time to reach the desired temperature levels was in some cases longer than the noted heat treatment. Thus rapidly raising the temperature as accomplished in an HTST unit could be evaluated along with the stability of the enzymes over longer periods of time. The effect of added hydrogen peroxide should also be studied because there is a limited use of this compound in treating cheese whey. Due to the effect of pH on the stability of residual milk clotting enzymes, requirements for heat destruction in Cottage cheese wheys should be checked. Another interesting study that could be undertaken would be to follow the activity of the residual enzyme present in the cheese as it cures. Differentiation of the milk clotting enzymes added at the time of cheese manufacture and the proteases formed during the curing process would be a challenge for whoever undertakes this area of study.
LITERATURE CITED


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James Winter Duersch

Candidate for the Degree of

Master of Science

Thesis: Heat Stability of Residual Milk Clotting Enzymes in Cheese Whey

Major Field: Nutrition and Food Science

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