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Persistence of *Mucor Miehei* Protease in Cheddar Cheese and Pasteurized Whey and its Effect on Sterile Milk Products

Randall Kirk Thunell
Utah State University

PERSISTENCE OF *Mucor miehei* PROTEASE IN CHEDDAR CHEESE AND PASTEURIZED WHEY AND ITS EFFECT ON STERILE MILK PRODUCTS

by

Randall Kirk Thunell

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ABSTRACT

Persistence of *Mucor miehei* Protease in Cheddar Cheese
and Pasteurized Whey and its Effect on Sterile Milk Products

by

Randall Kirk Thunell, Master of Science

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

Whey from a commercial cheese plant, taken at draining on five separate days, from cheese made with a *Mucor miehei* coagulant was cooled within 1 h to 4 C. Portions were adjusted from pH 4.2 to 6.4 at .2 pH intervals and subjected to HTST pasteurization at 73.9, 76.6, and 79.5 C for 25 sec. Milk clotting activity in whey was determined before and after pasteurization. Resistance to heat inactivation increased with decreasing pH. All measurable activity was destroyed above pH 5.4 by pasteurization at 79.5 C, above pH 5.8 at 76.6 C and above pH 6.0 at 73.9 C.

Milk clotting activity in Cheddar cheese made with *Mucor miehei* remained unchanged for 26 weeks.

Four commercial sterile liquid-milk-based products consisting of infant formula, concentrated infant formula, nutritionally complete food, and diet food were aseptically inoculated with sterile *Mucor miehei* protease solutions to concentrations ranging from 5×10^{-3} to 1×10^{-7} chymosin units/ml of product. The samples were stored at 30 C. After 20 weeks there was no change in the nutritionally complete food. The

diet food showed slight whey separation and thickening at 1×10^{-4} CU/ml and coagulation at higher concentrations. The infant formula showed definite whey separation and thickening at 1×10^{-4} CU/ml and coagulation and higher concentrations. The concentrated infant formula showed visible thickening at 1×10^{-3} CU/ml and coagulation at higher concentrations.

(76 pages)

INTRODUCTION

Dried whey is used as an ingredient in baked goods, dry mixes for pancakes, soups, puddings, cakes, frozen desserts, confections, beverages, meat emulsions, and whey-soya blends (8, 14, 15, 31, 57, 61, 62, 71, 72, 78, 88, 102, 112, 125, 132). Its use in liquid milk-based products however, is occasionally hampered by residual milk-clotting enzymes in the whey which produce casein coagulation. These enzymes remain in the whey following the normal manufacture of cheese.

Chymosin (rennin), an enzyme extracted from the abomasum (fourth stomach) of unweaned dairy calves, was the primary milk-clotting enzyme used in cheese production up until 1965, when the supply of chymosin no longer met cheese making demands. Cheese production in the United States increased from 1.5 billion pounds in 1960 to 2.9 billion in 1974 (7) while calf slaughter decreased from 8.22 million to 2.98 million annually (6). Due to higher meat prices, dairymen found it advantageous to raise their calves to maturity. The chymosin supply in the U.S. in 1973 met only 34 percent of the cheese making requirements (91).

Scarcity and the higher cost of chymosin led to a search for other milk-clotting enzymes. By 1965, porcine pepsin and chymosin-pepsin blends were the major enzymes used in cheese production, and by 1972, FDA regulations permitted the use of milk-clotting enzymes derived from controlled fermentations of the fungi *Endothia parasitica, Mucor pusillus* var. *Lindt,* and *Mucor miehei* (5).

Holmes and Ernstrom (54) showed that the milk-clotting enzyme from *Mucor miehei* survived the cheese making process better than

chymosin or pepsin. Duersch and Ernstrom (27) demonstrated that the *Mucor miehei* enzyme exhibited greater heat stability than the *Mucor pusillus* protease, bovine pepsin, *Endothia parasitica* protease, and procine pepsin. They further concluded that an increase in pH produced a decrease in heat stability of the enzymes with the exception of *Endothia parasitica* protease, which showed the opposite relationship.

The purpose of this study was to determine the high-temperature short-time pasteurizing temperatures needed to inactivate residual *Mucor miehei* protease in whey at various pH values. Another purpose was to determine allowable levels of residual milk-clotting enzymes in commercial milk-based products which use whey as an ingredient. A further objective was to measure the levels of *Mucor miehei* protease in cheese curd and whey and determine its survival during cheese ripening.

REVIEW OF LITERATURE

Milk-clotting Enzymes

Under suitable conditions, all proteolytic enzymes will clot milk (11, 110, 120). Milk-clotting enzymes have been obtained from almost every class of living organism (39). Chymosin (rennin) has long been the favored milk-clotting enzyme for cheese making, but due to scarcity and increasing cost, other milk-clotting enzymes have been substituted. Chymosin, however, remains the standard against which all others are evaluated. Chymosin as well as other milk-clotting enzymes acts on the micelles in milk to cleave a soluble macropeptide from k-casein to form insoluble para-k-casein (21). This cleavage destabilizes the casein micelle and causes the casein to clot in the presence of calcium.

Rennet (EC 3.4.23.4)

Chymosin (rennin) in a crude extract form called rennet, is derived from suckling calf abomasa. The name chymosin was first introduced by Deschamps (25) in 1840. Chymosin was still used in the literature as late as 1938 (133). The term rennin was introduced about 1890 (76) and was adopted as the name of the enzyme by several workers about 1930, and became generally accepted by all scientists up to 1970 when Foltmann (43) proposed that the name of the enzyme be changed back to chymosin to avoid confusion with the renal enzyme renin which is associated with hypertension. Chymosin has now been

adopted as the official name of the enzyme and given the identification number EC 3.4.23.4 (36).

Kleiner and Tauber (65) showed that chymosin originates in the stomach mucosa as a zymogen called prochymosin, which, upon contact with stomach acid, is converted to active chymosin. Premium quality chymosin from 10-30 day-old calves contains up to 12 percent pepsin activity (90). In 1940 Struble and Sharp (117) reported a zone of maximum chymosin stability centering at pH 4.0. Other workers since have found this observation erroneous. Mickelsen and Ernstrom (82) reported maximum chymosin stability from pH 5.0 to 6.0. Cheeseman (17) found maximum enzyme stability at pH 5.4. At pH 3.8, 30 C and at an ionic strength of 1.0, chymosin is more stable in sodium citrate, sodium lactate, and ammonium sulfate than in sodium chloride or potassium chloride (82).

The isoelectric point of chymosin is pH 4.45-4.65 (49). Foltmann (42) found enzymatic activity loss due to pH greatest between pH 3 and 4 and above pH 6.5. Activity loss below pH 4.5 was due in part to autolysis (82, 84). Activity loss above pH 6.0 increased with rising pH and temperature (82). According to Mickelsen and Ernstrom (82) the presence of chloride ions accelerates activity losses of chymosin at pH values below 4.5. Increases in ionic strength from 0.03 to 1.0 with NaCl at pH 3.8 resulted in an enzyme activity decrease from 65 to 30 percent.

Hamdy and Edelstein (48) found that the clotting activity of chymosin increased only slightly when the temperature was raised from 35 C to 45 C. At pH 5.1 milk-clotting activity was 4.5 times greater than at pH 6.3 (73). In the presence of hydrophobic substances

such as urea, enzymatic activity is rapidly and irreversibly lost (17, 126). Van de Beek and Gerisma (126) showed that the presence of sucrose and lactose stabilize chymosin against heat denaturation, even in the presence of urea by lowering the hydrogen-bond rupturing capacity of the medium.

Approximately 5 to 7 percent of the total rennet added to milk for cheese making is recovered in the cheese (29, 54). Dulley (29) reported milk-clotting activity of residual chymosin throughout cheese ripening. Holmes and Ernstrom (54) showed that the distribution of rennet between curd and whey is pH dependent, with more rennet being bound to the curd as pH decreases. At pH 6.8 rennet is largely released from the curd (56). Wang (129) reported even distribution of rennet between curd and whey at pH 6.1. When the pH was raised to 6.6, rennet bound to curd decreased to 36 percent. As pH was lowered to 5.7, 60 percent was bound to the curd.

Chymosin at sufficiently high concentrations has considerable proteolytic activity (29). Dulley (29) showed that a combination of rennet and starter caused a greater release of amino acids from casein than did starter or rennet alone. Edwards and Kosikowski (32) and Edwards (33) found that chymosin preferentially hydrolyzed α-casein. Itoh and Thomason (59) reported that among the major casein components the greatest production of non-protein nitrogen (NPN) by chymosin was from k-casein (9 percent) followed by α-casein (3 percent) and β-casein (2 percent). Sardinas (110) stated that chymosin hydrolyzes α-casein to a greater extent that it does β-casein. This is in agreement with Itoh and Thomason as α-casein is comprised of α_s-casein and κ-casein (122). According to Creamer (20) chymosin is more specific than pepsin in

its reaction on β-casein. Chymosin hydrolysis of β-casein proceeds very slowly (127). Fox and Walley (44) reported bitter flavors in chymosin hydrolysates of β-casein, but none in α_s. Pelissier and Manchon (98) reported chymosin hydrolysates of both α_{s1} and β-caseins to be bitter, with those of α_{s1}-casein being most bitter.

The rate of proteolysis of α_{s1}-casein by chymosin is fastest in the presence of 5 percent NaCl (44) and proteolysis of β-casein by chymosin or pepsin is almost completely inhibited in the presence of 10 percent NaCl and is reduced significantly by 5 percent NaCl (pH values 5.2 and 6.0; 4 C for 4 days, 32 C for 1 day). Electrophoretic studies show that k-casein is acted upon by chymosin and pepsin at NaCl levels as high as 20 percent but at 20 percent NaCl complete hydrolysis of k-casein does not take place. In the presence of 20 percent NaCl 50 percent of α_{s1}-casein was hydrolyzed by chymosin (44). Chymosin has limited and specific proteolytic activity on k-casein (118). Very low amylase activity of chymosin was reported by de Koning et al (68).

Pepsin (EC 3.4.23.1)

Porcine pepsin is obtained as an inactive zymogen, pepsinogen, which is activated at pH 2 to 3. If activation occurs at pH 3.0 or 3.9 several additional active pepsin species can be obtained (101). Mickelsen (84) showed that porcine pepsin undergoes rapid and complete inactivation within 15 minutes at pH 6.95 and 30 C. The enzyme loses activity in neutral or alkaline solutions according to Sardinas (110). O'leary and Fox (96) found that porcine pepsin concentrations

capable of clotting 2 ml of Berridge substrate in 4 minutes at 30 C was completely denatured in 3 minutes at pH 7.0 and 30 C. Bovine pepsin, on the other hand, lost only 5 percent of its activity under the same conditions. Normal cheesemaking concentrations of porcine pepsin are relatively stable below pH 6.0 (110), but will not clot milk in two hours above pH 6.7 because of enzyme instability (38, 90).

The distribution of porcine pepsin between curd and whey is similar to rennet according to Holmes and Ernstrom (54). As pH decreases, more pepsin is bound to the curd. Like rennet, porcine pepsin can be released from the curd at pH 6.8 but at pH values between 6.6 and 6.8 the enzyme is partially inactivated because of its instability at pH values approaching neutrality (56). Holmes and Ernstrom (54) further found that during cheddar cheese making less than half of the pepsin added to milk was recovered in curd and whey, and no pepsin was retained in pressed curd.

Green (45) found chicken pepsin unsuitable for cheese manufacture. The cheese had a poor body and weak Cheddar flavor with intense off-flavors. Cheese made with porcine and bovine pepsins was only slightly inferior in quality and flavor to rennet cheese.

Early applications of adult bovine extract (ABE) as a milk coagulant proved unsuitable because of cost (90, 45). Enzyme yield was lower than from calf mucosa and up to eight times as much tissue had to be extracted for each enzyme unit (90). Today, adult bovine extract is being economically extracted from the stomach mucosa of older animals.

Porcine pepsin was not an ideal milk coagulant because of its

sensitivity to changes in pH of milk (19, 59, 105) and weaker proteolysis in cheese than chymosin (59). Milk clotting time increases at pH values above 5.3 (38). The following shortcomings of porcine pepsin have been listed: extended setting time, weak curd, loss of fat in whey, bland flavor, limited use above pH 6.5 because of activity loss and defects in texture and consistency. Pepsin produces good quality cheese when combined 50:50 with rennet or microbial coagulants (22, 45, 90, 110).

Chicken and porcine pepsin exhibit more proteolytic activity on casein at pH 6.3 than chymosin or bovine pepsin (45). Maximum proteolytic activity of porcine pepsin on hemoglobin is at pH 1.8 (85). At pH 9.0 the proteolytic activity of adult bovine extract on casein was evident, whereas none was exhibited by porcine pepsin or rennet (106). De Koning et al (66) found porcine pepsin very low in amylase activity (68).

Sodium chloride influences proteolysis of casein by porcine pepsin. In the presence of 15 and 20 percent NaCl at pH 5, a broad spectrum of high mobility polypeptides is produced by pepsin. This is not the case with rennet (44). At pH 6.5, such levels of salt have an inhibiting effect on pepsin proteolysis. Porcine pepsin acts upon k-casein at NaCl levels as high as 20 percent but k-casein is not completely hydrolyzed at these higher salt concentrations. Proteolysis of β-casein is reduced by 5 percent NaCl and completely inhibited in the presence of 10 percent NaCl. The rate of proteolysis of α_{S_1}-casein is fastest in the presence of 5-10 percent NaCl (44).

Chymosin-porcine pepsin blends

Porcine pepsin produces good quality cheese when combined with rennet or microbial proteases (22, 45, 90, 110).

Mickelsen and Ernstrom (85) found maximum enzyme stability in blends at pH 5.5. Porcine pepsin becomes unstable above this value, while chymosin is attacked by pepsin below this value. In the presence of 25 percent or more porcine pepsin at pH 3.0, chymosin undergoes rapid and complete destruction in 48 hours (84,85). As the pH decreases from 5.5 to 3.0, destruction rates also increase (85). The presence of porcine pepsin at pH 3.8 and 4.8 accelerates chymosin activity losses which are proportional to the amount of pepsin present (84, 85).

Chymosin is most stable in blends between pH 5.5 and 6.5 whereas porcine pepsin is most stable between pH 3.8 and 5.5 (85). Mickelsen and Ernstrom (85) reported porcine pepsin activity losses at pH 6.0 with greater losses occurring as the pH increased. At pH 7.3 and 30 C , porcine pepsin undergoes complete inactivation in 48 hours while chymosin is only slightly affected (84, 85). Chymosin may have a slight stabilizing effect on pepsin at pH 6.5 (85). At pH 3.0 and 6.5, chymosin showed no evidence of destroying porcine pepsin activity.

Holmes and Ernstrom (54) found the enzyme distribution of rennet-pepsin blends between curd and whey to be similar to that of chymosin, except at pH values approaching neutral where pepsin becomes unstable.

In cheesemaking trials, Emmons et al (34) made cheese using a 50:50 rennet-porcine pepsin blend, and rennet alone. When compared with the rennet cheese, rennet-pepsin blend cheese had slightly poorer texture after 3 weeks, but showed no significant difference after 6 and 12 months. Flavor scores for the mixture cheese were slightly higher than rennet cheese scores after 12 months, but Cheddar flavor in the mixture cheese was slightly less intense than

in rennet cheese. Cheese made with blends of rennet and porcine pepsin were slightly firmer than chymosin cheese at 6 and 12 months, with no differences in pH, moisture content, or fat in dry matter noted.

In other trials (35) 10 vats of Cheddar cheese were made using blends of 94 percent chymosin plus 6 percent bovine pepsin and blends of 6 percent chymosin and 94 percent bovine pepsin. Blends of 94 percent bovine pepsin gave significantly lower cheese yields with high protein and fat losses in the whey when compared with the 94 percent chymosin blend. Flavor and texture of both blends, however, were similar. Proteolysis was less extensive in the 94 percent bovine pepsin cheese than in the cheese made with 94 percent chymosin (34).

Microbial proteases

Rennet substitutes of microbial origin are of particular value because of their unrestricted availability (109), ease of propagation in unlimited quantities (90), and their various properties which permit a selection for the most suitable property for a particular cheese (115).

In the search for suitable rennet substitutes, over 380 bacteria and 540 fungi were examined (67, 109). Only proteases from the fungal species *Endothia parasitica*, *Mucor miehei*, and *Mucor pusillus* var. *Lindt* proved acceptable for large-scale commercialization (110). FDA regulations (5) permit commercial cheese production using milk-clotting enzymes derived from pure culture fermentation of four microorganisms: *Bacillus cereus*, *Endothia parasitica*, *Mucor pusillus* var. *Lindt*, and *Mucor miehei*. Most microbial proteases proved unsuitable for cheese production because they contributed to off-flavors or bitterness

(63, 74, 99, 100, 109), excessive acid (109), inferior body and texture (100, 109), and excessive proteolysis (13, 50, 100, 109, 121, 127) in the cheese.

Endothia parasitica (EC 3.4.23.10). A milk-clotting enzyme (EP protease) derived from the fermentation of *Endothia parasitica* appeared in 1967 as one of the first commercial microbial proteases (110) for cheese making. *Parasitica* is a unique species of the genus *Endothia*. Of the other 6 species of *Endothia*, none synthesizes significant amounts of milk-clotting enzyme, while all 8 strains of *parasitica* clot milk in a similar manner (109, 110). EP protease exhibits maximum stability in water at pH 4.5 (74, 109, 110) and can be destroyed by heating at 60 C for 5 minutes (109, 110). At 50 C, only 30 percent activity is lost after 30 minutes and at 24 C the enzyme is stable from pH 3 to 6.5 for 3 hours (74). Rapid inactivation of EP protease due to autolysis occurs below pH 2.5 (68, 74) and at pH 5.5 to 6.5 enzyme denaturation results in decreased solubility and loss of activity (87). The isoelectric point of EP protease is pH 5.5 (109, 110). At pH 7 and above, there is no enzymatic activity present (13). The rate of autolysis of EP protease is increased at pH 2.5 by the addition of NaCl or KCL (74). Sardinas (109) found chymosin and EP protease to have comparable milk-clotting activities between pH 6.0 and 7.0. Chymosin, however, is more active below pH 6.0. Enzyme activity of EP protease at pH 5.1 is 2.5 times greater than at pH 6.3.

Low concentrations of urea will denature EP protease at pH 2.5 and 6.5, but at pH 3.5 the enzyme is stable to urea (74).

Sensitivity of EP protease to substrate pH is much less between pH 5.1 and 6.5 than for chymosin (73). Larson and Whitaker (73) reported the enzymatic phase to be the rate determining step in milk coagulation by EP protease.

Endothia parasitica protease possesses particularly strong proteolytic properties (13, 83, 100, 127). Specificity, as determined on the oxidized B-chain of insulin, is broader and different than that of chymosin or porcine pepsin (73). Proteolytic activity of EP protease is higher at all pH values than *Mucor*-derived proteases, chymosin, or porcine pepsin (83, 100, 118), and preferentially hydrolyzes β-casein (32, 99, 103, 127). Tam and Whitaker (117) found that EP protease hydrolyzed α-casein to a greater extent that it did β-casein. Greater hydrolysis also occurred at pH 5.5 than at pH 6.0. β-casein hydrolysis is lower at pH 3.0 than at pH 3.5. Vanderpoorten and Weckx (127) reported lower proteolytic activity of EP protease than other enzymes on k-casein. Using electrophoretic patterns, Larson and Whitaker (73) showed that EP protease caused more k-casein fragmentation than did rennet or pepsin, particularly at pH 2.5. Dekoning, et al (68) reported very low amylase activity for EP protease.

Of the microbial proteases, EP protease is the least sensitive to calcium variations, and is less sensitive than chymosin (48, 110). Increasing ionic strength at pH 6.5 stabilizes EP protease but decreases stability at pH 2.3 (74).

Additions of 0.075 percent $CaCl_2$ to milk cut milk clotting time by EP protease in half, and at various $CaCl_2$ concentrations, the milk-clotting activity of EP protease parallels rennet activity. Coagulation time of milk with EP protease is reduced by 14 percent when 0.2 to 0.4 percent NaCl is added to milk (48). An addition of KCL to milk increases

clotting time but decreases proteolytic activity of EP protease on milk (73).

Endothia parasitica protease is still offered for sale in the U.S. but *Mucor miehei* strains have taken over most of the market. It was reported that one problem with EP protease was its tendency to produce mealy body and bitter taste in long-hold Cheddar cheese. For practically all other types of cheese including short-hold Cheddar, EP protease presented no problem (personal communication, J. Shovers to G. H. Richardson, March 2, 1973). It was later shown that good quality Swiss cheese could be made with EP protease. Raps, et al (103) reported slightly higher curd firmness in milk with an EP protease-chymosin mixture than with chymosin alone. Alais and Novak (2) had good results in the manufacture of several types of cheese made with EP protease and recommended it as a suitable chymosin substitute. Shovers and Bavisotto (111) also found EP protease suitable as a partial or complete chymosin substitute. Cheese made with EP protease had acceptable body and flavor development and was graded equal or superior to rennet cheese. Accelerated flavor and body development was noted in the EP protease cheese. Nelson (91) suggests that use of EP protease be limited to Swiss cheese because of bitter flavor development in some cheese varieties. Bitter flavors in cheese made with EP protease have also been reported by Poznanski et al (100) and Phelan (99).

Mucor pusillus var. Lindt (EC 3.4.23.10). Milk-clotting enzyme (MP protease) derived from the controlled fermentation of the fungus *Mucor pusillus* var. *Lindt* appeared commercially about the same time as *Endothia parasitica* protease (110).

Richardson et al (105) reported severe activity losses when MP protease was combined and stored with liquid rennet. The more rennet that was present, the greater the loss of activity, suggesting that MP protease destroys rennet activity. When MP protease was added to calf rennet at low concentrations, a synergistic effect was observed which greatly increased milk-clotting activity over that of the separate solutions (124).

MP protease is more sensitive than rennet (between pH 6.4 and 6.8) but less sensitive than pepsin (105). Brandl (13) demonstrated enzyme activity even at pH 8.1. In the pH range 5.5 to 6.2, fungal enzyme stability was better than rennet. MP protease has greater thermostability than rennet or pepsin at all pH values below pH 7. At pH 7.0 the enzyme can be destroyed by heating at 68.3 C for 15 seconds (27, 110). MP protease has an isoelectric point of pH 3.5 to 3.8 and exhibits maximum stability at pH 5.0. Milk was most actively coagulated at pH 5.5 (110). The optimum temperature for milk coagulation by MP protease was between 42-45 C (103).

MP protease is stimulated more by trivalent than by divalent ions (124). An addition of 0.4 to 0.6 percent NaCl to milk will reduce coagulation time by MP protease by 36 percent (48). Calcium ions greatly stimulate the milk-clotting activity of MP protease (48). Milk-clotting time was reduced by half by the addition of 0.04 percent $CaCl_2$. Milk-clotting activity of MP protease is more calcium sensitive than that of rennet and is the most calcium sensitive of the fungal proteases (48).

Proteolysis in cheese made with MP protease is more extensive than when made with calf rennet (13, 59, 100, 105, 118), and correlates with the amount of enzyme added (92). Tam and Whitaker (118) found that the extent of MP protease hydrolysis in casein fractions to be in order of α, κ, and β-casein. Hydrolysis of β-casein by MP protease is much greater at pH 5.5 than at pH 6.0 (13, 118). Green and Stackpoole (46) reported an increase in α_s-casein breakdown with increasing proportions of MP protease in a MP protease-porcine pepsin mixture. According to Edwards and Kosikowski (32) α and β-casein fractions are attacked to about the same degree by MP protease. The fungal enzyme has a high milk-clotting activity to proteolytic activity ratio (110). Lipase activity of commercial MP protease preparations is higher than that of calf rennet (13, 105) and amylase activity is very high (68, 69).

Cheese made with MP protease had a slightly bitter flavor during ripening and when aged 14 months (63, 100, 105). Kikuchi and Toyoda (63) reported lower cheese yields and a more brittle curd at cutting with MP protease. After cooking, there was no significant difference when compared to rennet curd. *Mucor pusillus* protease-porcine pepsin mixtures resulted in lower cheese yields when compared with rennet (45). Nelson (89) found no significant differences in cheese yields using MP protease alone, MP protease-rennet, and MP protease-pepsin blends. Nielsen (92) suggested modifying the cheese making process slightly when using MP protease by adding 0.015 percent $CaCl_2$ to the milk and prolonging the setting time by 10-15 minutes. Nielsen reported a lower moisture content in MP protease cheese and increased whey fat losses have also been noted (46, 105). Poznanski

et al (100) agree that MP protease is as good as rennet for cheese production. Nelson (89) made cheese comparable to rennet cheese using MP protease alone and in combination with rennet or pepsin. All cheeses had acceptable quality throughout curing; some showed rapid body breakdown.

Green and Stackpoole (46) supported these findings and reported hastened Cheddar flavor development resulting from blends with high MP protease proportions. MP protease-porcine pepsin blends produced a more acceptable cheese than either coagulant alone. Flavor and acceptability was similar in cheeses made with 30-40 percent MP protease when compared to rennet (46).

Distribution of MP protease between curd and whey is not pH dependent. Approximately 3 percent of the coagulant added to milk is retained in the curd (56). The proportions of MP protease retained in pressed cheese was significantly lower than calf rennet (54).

Mucor miehei (3.4.23.10). A milk-clotting enzyme (MM protease) has been derived from the pure culture fermentation of the fungus *Mucor miehei*. Federal regulations were ammended in April of 1972 to add MM protease to the list of acceptable fermentation derived milk-clotting enzymes (5). Under the U.S. Federal Food Drug and Cosmetic Act (41) MM protease may be used in commercial cheese production.

The *Mucor*-derived enzyme is an acid protease (97, 115) of similar amino acid composition to the *Mucor pusillus* protease and to chymosin (115). No serine or sulfhydryl groups are found at the active center (115). Using gel filtration, three coagulation-active fractions have been separated (30). Fractions one and three are coagulation-active

at 18 C while fraction two is inactive at 18 C but most active at 35 C Oka et al (94) report MM protease specificity almost identical with MP protease against synthetic peptides and the oxidized B-chain of insulin.

The optimum stability range for MM protease is pH 3 to 7 (81). In acetic acid and citric acid buffers, maximum thermal stability of MM protease is at pH 4-4.2 (77). Phelan (99) reported less sensitivity for MM protease than rennet in the pH range 6.6-6.8. Nelson (91) stated that *Mucor*-derived milk clotting enzymes are more pH sensitive than rennet, but less sensitive than pepsin. *Mucor miehei* protease is stable for 24 hours at 40 C over a range of pH 1 to 7.5 using casein, bovine serum albumin, and urea and acid denatured hemoglobin substrates (115). After 60 minutes at pH 9.0 and 20 C MM protease and MP protease lost no activity while rennet was completely denatured. MM protease suffered no activity loss at pH 6.0 and 60 C for 20 minutes, whereas MP protease and rennet were completely inactivated (96). Ledward et al (77) reported the isoelectric point of MM protease at pH 4.0-4.2.

Coagulation time for MM protease is cut in half by a temperature elevation from 37 C to 42 C (49). Duersch and Ernstrom (29) showed that MM protease was more heat stable than MP protease, rennet, or pepsin at all pH values. This was supported by Hyslop (59). Duersch and Ernstrom further showed that as pH decreases, the heat stability of the enzyme increases. *Mucor miehei* protease was destroyed by a 15 second heat treatment of 68.3 C pH 7.0 but at pH 5.2, it was still active after 10 minutes of heating at 73.9 C. Sternberg (115) found that the presence of 6.8×10^{-3} M $CaCl_2$ enhanced pH stability of MM protease over the pH range 1.0 to 9.0. Enzyme stability was measured over 24 hours at 40 C.

MM protease exhibits less proteolytic activity than other microbial coagulants (95). The optimum pH for proteolysis of denatured hemoglobin was 4.1-4.4 and pH 4.0 on B-chain oxidized insulin. Proteolysis of casein decreases by over half from pH 5.5 to pH 7.5 (115). Attack by MM protease on α and β-casein fractions is about the same as by MP protease (32, 33). Edwards and Kosikowski (32) made electrophoretic studies of proteolysis in Cheddar cheese made with rennet, EP protease, MP protease, and MM protease. Electrophoretic patterns were studied after 0, 4, and 20 weeks of curing at 10 C. They reported the order of proteolysis on α-casein to be rennet >*Mucor*>*Endothia*. For β-casein *Endothia*>*Mucor*>rennet. Phelan (99) reported β-casein breakdown by MM protease to be similar to that of chymosin but less extensive on α-casein. Proteolysis of k-casein is more rapid with proteases than with other microbial proteases (33). Sternberg (116) found the k-caseino glycopeptide cleaved during milk clotting and sialic acid release by MM protease to be similar to those released by chymosin during milk clotting. Phelan (99) states that MM protease is the least proteolytic of the commercial microbial proteases. When used alone or in 50:50 rennet-MM protease blends, MM protease shows similar proteolytic activity to chymosin. pH activity curves for proteolysis by MM protease on acid denatured hemoglobin resemble those of rennet (115). Martens (80) reported no increased lipolysis with MM protease.

Chymosin and MM protease possess similar calcium sensitivities (48). Coagulation time is reduced by half with the addition of 0.5 percent $CaCl_2$ and a similar addition of NaCl will reduce clotting

time by one-third. Nelson (91) reports that *Mucor*-derived enzymes are affected more by changes in free calcium concentration than is chymosin. Clotting and proteolytic activity can be partially inhibited by $HgCl_2$ and mercaptoethanol and completely inhibited by cyncal (46). Aluminum has little effect on proteolytic activity but it does prevent the breaking of bonds essential to milk clotting. Copper displays the same inhibitive effect but to a lesser degree.

In cheese making trials, Thompson (123) found MM protease to clot milk "identically" to quality rennet. MM protease has been used in the manufacture of a wide variety of cheeses (19, 123). Phelan (99) reports good quality cheese from MM protease. Clotting and cutting times for MM protease cheese as well as curd characteristics are similar to those of rennet and no modifications of the cheese making process are necessary to compensate for fresh or aged milk. Christensen (19) observed no bitterness in cheese during curing, even when increased amounts of microbial coagulant were used. Martens (80) described a less favorable flavor in Gouda cheese made with MM protease when compared to rennet-made Gouda. The flavor differed only slightly from rennet controls and was improved by decreasing the amount of enzyme used and adding $CaCl_2$. An occasional slight bitter taste was also reported. Dineson et al (26) compared MM protease with rennet in 12 vats of cheese in three different factories. MM protease gave slightly higher non-protein nitrogen (NPN) values in the whey and flavor scored lower than rennet after 3 weeks and 3 months. This difference disappeared after 6 months. No bitterness or increase in intensity of Cheddar flavor was noted after curing for 15 months. Martens (80) reported a softer body in Gouda cheese made with MM protease compared to

rennet cheese. Similar breakdown products in cheese made with MM protease and chymosin were also noted (19, 80, 123). Little difference in pH, moisture, or salt content of the cheese has been noted (80).

MM protease was found to neither stimulate nor inhibit cheese starter, and fat losses in the whey are minimal (123). Freezing does not affect enzyme activity (19).

Holmes and Ernstrom (54) found that EP protease retention in cheese curd is not pH dependent. The microbial protease apparantly doesn't bind to the curd.

Bacterial proteases

Proteases derived from bacterial sources have not found general use in cheese manufacture because of excessive proteolysis (59, 100, 103) soft curd, and bitter flavors (63, 100, 103).

Plant proteases

Rennet substitutes from most plants produce bitter flavors, defective textures (68) and possess strong proteolytic activities (33, 121). No substitutes from plants are commercially available.

Sterile milk products

Heat sterilization of concentrated milk products can cause multiple effects including serum protein denaturation, casein dephosphorylation, increases in acidity, salt equilibria changes, and interactions between lactose, casein, and other serum constituents (69, 97). Heat sterilization can also alter milk protein structure,

inhibit or prolong coagulation by rennet (52, 60, 108) or give to rise to a modified casein which is less calcium sensitive (3, 86).

Heat sterilization causes a transfer of soluble calcium to colloidal calcium (60, 97). Approximately 6 milligrams of calcium and 4 milligrams of phosphorous per 100 milliliters of milk are transferred by heat treating milk at 85 C for 30 minutes (60).

Parry (97) states that high temperature heat treatment, as well as enzymatic action, can cause hydrolytic cleavage of peptide and phosphate bonds in casein. During pasteurization, only minor changes in non-protein nitrogen (NPN) occur, but under rigorous heating, substantial hydrolysis and dephosphorylation of casein occurs. Degradation of the main casein components by sterilization was noted by Alais et al (1). Caseino-glycopeptides isolated from k-casein after heat treatment had very similar amino acid compositions to those released by rennet action. Nakaniski and Itoh (87) observed changes in the electrophoretic patterns of k-casein upon heating above 110 C Changes became more distinct as the temperature increased. The release of NPN and sialic acid from k-casein increased linearly with high temperature heating (87) and k-casein lost its ability to stabilize α_s-casein in the presence of calcium ions (3, 87, 136, 137). K-casein, in the presence of $CaCl_2$ at low concentrations (0.005 - 0.05M) is precipitated by heating at 100 C for 5 minutes (136, 137). At higher $CaCl_2$ concentrations (0.15M) precipitation of k-casein is completely prevented (137). Zittle (136) reports k-casein to be heat labile in 0.05 sodium chloride and calcium chloride.

Heat treated α_s-casein became more soluble in the presence of calcium (3). Alais et al (1) reported that heat degradation of α_s-casein

was extensive and resulted in more products than heat degradation of β or k-casein. β-casein appears to be the most resistant to degradation by heat.

One problem facing the sterile concentrated milk industry is thickening and eventual coagulation of the product during storage (66). Storage thickening and gelation of sterilized milk concentrates is accelerated at higher concentrations of milk solids (12, 97). Using electronmicroscopy, Board et al (12) noted a tendency for micelles to link together by protein strands in concentrated milk. Sterilization caused protein fusion in all of the whole milk samples tested. Cross-linking between protein chains was also found by Andrews and Cheeseman (3). Electron microscopy studies of protein structural changes in sterilized milk (23) showed denatured β-lactoglobulin becoming bound to casein micelles during the manufacture of concentrated sterile milk. They also showed the aggregation of micelles to form chain and branch-like structures which increased the viscosity of the milk. Sedimentation in sterile milk concentrates involves cross-bridging between proteins and can be minimized by increasing the pH (12).

In 1971, Andrews and Cheeseman (3) demonstrated the occurrance of a Maillard-type reaction during storage of ultra-high temperature (UHT) sterilized milk, that is capable of modifying casein and causing polymerization. They noted that ultra-high temperature processing of milk caused the production of highly reactive carbonyl compounds. Electrophoretic changes in casein were produced when milk was stored

at or above ambient temperature, but no such changes were produced at lower than ambient temperatures. Andrews (4) found that sterile milk stored for several months at 30 C and 37 C contained modified proteins which lacked the electrophoretic mobility of native α_s and β-caseins. He also noted that the extent of polymerization of caseins and whey proteins in UHT milk was dependent on both temperature and storage time. This fact is supported by Samuel et al (108). Andrews found casein and whey proteins covalently bound together in the following proportions after storage of UHT milk for six months: 50 percent at 37 C; 40 percent at 30 C; 26 percent at 20 C; 21 percent at 4 C. Polymerization may preferentially involve α_s and β-caseins, but β-casein reacts at a slower rate (4).

Wheelock and Hindle (130) showed a progressive decrease during storage for 7 months in the amount of carbohydrate attached to k-casein in heat sterilized milk. Samuel et al (108) found that the stability of UHT milk to ethanol, calcium, and rennet decreased with time, and the onset of gelation did not depend on the degree of protein breakdown, nor was proteolysis a major source of storage gelation. Samples of UHT milk gelled after storage for 13 months at 4, 20, and 30 C, but not at 37

Milk micelles remain in colloidal suspension after heat treatment, but low pH, heat, and increasing storage time all favor micelle aggregation. Cheeseman and Knight (18) found that increased storage time gave rise to an increase in the proportion of casein aggregates in UHT milk, but no corresponding increase in size of the casein aggregates was observed. Wilson et al (131) found a

decrease in titratable sulfhydryl groups in concentrated UHT milk during storage, and surmised that disulfide bonds are involved in gel structures.

Preheating milk before sterilization induced a high degree of stability (60). Samuel, et al (108) found negligible protein decomposition in milk samples autoclaved and then stored at 4 and 20 C. Significant protein decomposition did, however, occur at 37 C. Autoclaved milk stored at 4, 20, and 30 C was still fluid after 2 years.

Hindle and Wheelock (52) noted that preheating retards the secondary or coagulation phase of milk clotting by enzymes by decreasing free calcium in the milk. They determined that the primary phase of chymosin action on heat-treated milk was unaffected by the heat-sterilization process. Kannan and Jenness (60) reported an increase in coagulation time after heat-treating due to complexing of β-lactoglobulin with casein in such a way that it inhibits rennet clotting. They suggest that this heat-induced reaction interferes with the enzymatic action of rennet. More recently, Hindle and Wheelock (53) have shown that the primary (enzymatic) phase of milk clotting as measured by soluble peptides released in 12 percent TCA is partially inhibited because of a heat-induced decrease in the release of non-carbohydrate-containing peptides from k-casein. Heat-complexing of β-lactoglobulin and k-casein and inhibition of the enzymatic phase affects mainly the release of peptides lacking carbohydrate.

Heat sterilization of milk causes an increase in rennet coagulation time, which is further prolonged by holding the milk after heat treatment (52, 60). Prolongation of clotting time in skim milk is enhanced by adding 0.2 percent β-lactoglobulin (60). Kisza et al (64) found an

increase in heat stability when 0.05 to 0.6 percent k-casein was added to milk and a decrease with additions of β-lactoglobulin and α-lactalbumin which complex with casein, thus also interferring with enzymatic action (60, 97).

Thickening of condensed milk can be retarded by additions of citrate and phosphates (66). Gelation is delayed when polyphosphates are added to concentrated milk before sterilization (97, 131) whereas orthophosphates speed gelation (131). Added phosphates chelate soluble calcium in the milk and alter the ionic character of the micelle surface thus reducing the tendency for whey proteins or gel proteins to bind on the micelle surface. Browning and undesirable flavors occur prior to gelation when concentrated milk containing 0.05 percent added polyphosphate is stored at 37.8 C. An increase in viscosity of concentrated milk is observed with polyphosphate additions. Klepacka and Pijanowski (66) reported that after sterilization of condensed milk, tri- and higher polyphosphates are hydrolyzed to stable pyrophosphate forms. After 8 months storage of condensed milk at 30 C, samples with tri- and tetra-phosphates showed no increase in viscosity but samples containing diphosphates were practically coagulated. It is proposed that the stabilizing action of phosphates takes place prior to or during sterilization of condensed milk.

The heat stability of milk depends largely on its pH, with maximum heat stability exhibited at pH 6.7 (69, 133).

Residual milk-clotting eyzymes in whey

Total U.S. production of dried whey in 1974 exceeded 851 million pounds. Of this, 453 million pounds were utilized for human consumption representing an increase of 18 percent over 1973 (7). Because microbial

proteases possess higher heat stabilities than animal rennets, active residual milk-clotting enzymes may be present in dried or concentrated whey, thus limiting their use in many milk-based products.

Harper and Lee (51) encountered proteolysis of whey proteins caused by residual milk coagulants while producing undenatured whey protein concentrates. This led them to further investigate residual milk-clotting enzymes in whey. Whey samples containing rennet, pepsin, rennet-pepsin blend, and microbial rennets from *Mucor miehei* and *Mucor pusillus* var. *Lindt* were collected, divided into two portions and the pH adjusted to 6.6 and 4.5 respectively. The samples were then stored at 2 C and enzyme activity followed daily. After 16 days, no activity losses were observed at pH 4.5 and only pepsin showed an activity loss (30 percent) after 16 days of storage at pH 6.6. When whey samples were pasteurized at 62.8 C for 30 minutes at pH 6.6, activity losses for chymosin, pepsin and rennet-pepsin blends were greater than 98 percent. Activity losses for *Mucor*-proteases were 93 to 98 percent. At pH 4.5 all of the coagulants retained 75 to 85 percent of their activity. Harper and Lee (51) found that pH drops of 0.2 units resulted in measurable increases in rennet and MM protease activity. This fact was verified by Duersch (28).

High temperature short-time pasteurization of fluid whey at pH 6.3 resulted in 90 percent inactivation of rennet. Whey concentrated by low-temperature ultrafiltration still had 91 percent residual rennet activity, and 65 percent activity after concentration and pasteurization (51). Spray drying pasteurized concentrates at an inlet temperature of 218.3 C reduced residual rennet activity to 37 percent. Pasteurization followed by forewarming at 82 C for 10 minutes,

then vacuum concentration, eliminated residual activity of rennet. Harper and Lee found 92 percent inactivation of all coagulants by adjusting the whey to pH 6.6 prior to high-temperature short-time pasteurization. Verhey (128) spray dried concentrated whey to which rennet was added. In all cases there was enzyme activity in the powder. Unchanged rennet activity was also observed in dried unpreconcentrated whey.

Measuring milk clotting activity

Milk clotting is a two-phase process. The primary or enzymatic phase involves the cleavage of a macropeptide from k-casein which has a destabilizing effect on the milk micelle complex. The altered casein then aggregates during the secondary or non-enzymatic phase. Variations in milk composition and added salts affect this phase (39).

Sommer and Matsen (113) devised an apparatus to study rennet coagulation of mastitic milk. Bottles containing milk samples were rotated in a water bath to produce a thin milk film on the inner surface of the bottle. Coagulation was marked by the sudden fracture of the milk film and coagulation time was recorded in bottle revolutions.

Because of natural variations in milk composition, clotting times vary. Berridge (9) found that by reconstituting 60g low-heat non-fat dry milk in 500 ml 0.01M $CaCl_2$ he was able to both shorten the time lag between phases and eliminate some of the variations caused by varying milk composition, thus giving more reproducible clotting times. Test tubes containing 10 ml of substrate were tempered in a 30 C water bath for 30 minutes. One ml of diluted chymosin was added to the substrate. A stirring rod was dipped into the substrate and touched to the side of the tube to produce a flowing milk film.

The end point was observed as the milk film fractured and enzyme activity was measured as the time elapsed from enzyme inoculation to film fracture.

Berridge (10) was later able to automate his method by fitting the test tube to a rubber bung. This enabled him to keep the milk film flowing at an optimum rate, and eliminated the need for constant dipping with a stirring rod. The tube was immersed at a 30 degree angle in the water bath to keep the milk film continually below the surface. Clotting times using this improved method were more reproducible than the old method.

Ernstrom (37) used the Berridge substrate and Sommer-Matsen apparatus in combination to measure rennet activity. Testing was carried out at 30C on 25 ml portions of the substrate and the first appearance of visible flecks on the moving glass surface constituted the end point. Standard rennet was arbitrarily given a value of 100 chymosin units per milliliter (CU/ml) and had an activity such that 1 ml of a 1:250 dilution would produce visible substrate clotting in 100±5 revolutions. Activity of the unknown enzyme solution was related to that of the standard.

DeMan and Batra (24) adapted an automatic blood clot timer for measuring milk clotting time. This instrument has the advantage of being fast, uses less substrate, and detects the end point automatically. The instrument, however, has a higher coefficient of variation (CV) than the Sommer-Matsen apparatus. (Gene Hong, personal communication).

Cheeseman (16) studied the diffusion of rennet and other proteolytic enzymes in casein-agar gels. Characteristic precipitation zones for each milk clotting enzyme were formed as the enzyme diffused

through the gel. When measured after 48 hours, the mean diameters of the zones were related to enzyme concentration. The sensitivity of this method, however, was poor and did not compare favorably with Berridges' method.

A diffusion slide with a thin layer of calcium caseinate agar was used by Lawrence and Sanderson (75) to quantitate milk clotting enzymes at very low concentrations. The number and width of the precipitation zones was a function of the enzyme used and the diameter of the zones, as measured with Vernier calipers, was related to concentration. Humidity control of the incubating slide and extreme care in slide preparation were required with this method.

Everson and Winder (40) determined rennet coagulation time by adapting an instrument for continuous measurement of the velocity of ultrasonic energy through a milk substrate. Coagulation was marked by a sharp increase in sound velocity which was recorded automatically as the end point.

Richardson et al (106) and Kopelman and Cogan (70) continually monitored milk viscosity by means of a rotational viscometer to measure milk clotting activity. The instrument accurately detects and records a sharp increase of viscosity as coagulation occurs.

Reyes (104) measured residual rennet activity in curd and whey using a sensitive substrate proposed by Gorini and Lanzavecchia (47) that was modified by Wang (129). This substrate was 12-15 times more sensitive than the Berridge substrate. Non-chymosin substances in whey, however, affected coagulation time.

Holmes (56) developed a linear diffusion test for estimating low concentrations of milk clotting enzymes by utilizing a casein-agar

substrate in sedimentation tubes. The test substrate contained 0.5 percent whole casein, 3.6 percent sodium acetate, 0.1 percent $CaCl_2$ and 0.7 percent Ion Agar. The pH of the substrate was adjusted to 5.7 with 0.1M HCl. Concentrations of 1×10^{-4} to 1×10^{-1} CU/ml were detected with a standard deviation of 5 percent. Concentrations lower than 1×10^{-4} were either undetectable or deviated from a straight line relationship between diffusion distance and concentration. When testing concentrations of 10^{-1} CU/ml or higher, the conventional clotting test with Berridge substrate was more precise and convenient. Salt concentrations up to 3 percent and whey solids had no significant effect on the diffusion assay. The linear diffusion test was approximately 60 times more sensitive than the radial diffusion test of Lawrence and Sanderson (75) and 300 times more sensitive than the assay of Reyes (104). Holmes lists the advantages and improvements of his test as greater sensitivity than previous methods of activity analysis, allows for multiple assay, no special equipment for humidity control needed, tubes can be prepared in advance and stored in a refrigerator, diffusion distance can be easily measured with a densitometer, and whey solids and salt up to 3 percent have no significant effect on enzyme diffusion rate thus allowing direct measurement of enzyme activity in whey and curd slurry filtrates. Some disadvantages noted are: 48 hour test time, occasional clouding of the casein-agar gel, and the requirement for a high quality casein.

Duersch (28) modified Holmes' substrate to eliminate clouding and to sharpen diffusion bounderies in the casein-agar gel. This was achieved by eliminating calcium chloride, replacing whole casein

with k-casein, and raising the final pH to 5.9. Duersch's substrate contained 0.54 percent k-casein, 3.6 percent sodium acetate, 0.73 percent Bacto agar and 95.13 percent water. Diffusion distance was measured with a densitometer and plotted against a standard curve to determine concentration. Using the linear diffusion test with the modified substrate, Duersch studied the heat stability of residual milk clotting enzymes in whey.

METHODS OF PROCEDURE

Collection of whey

Liquid whey from Cheddar cheese made with *Mucor miehei* protease was collected at dipping from vats at Cache Valley Dairy Association, Smithfield, Utah. Whey was collected in 38 liter (10 gal.) milk cans and transported without refrigeration to the Utah State University Dairy Lab. Upon arrival, the whey was mixed in a large vat with agitation and cooled to 4 C for overnight storage.

High-temperature short-time processing of whey

The temperature of the whey was raised to 22 C with agitation to insure uniform heating. The temperature was held at 22 C throughout processing. The pH of the whey was determined with a Corning digital pH meter model 110. The whey was adjusted to pH 6.2, 6.0, 5.8, 5.6, 5.4, 5.2, 5.0, 4.8, 4.6, 4.4, and 4.2 by adding 37 percent hydrochloric acid or 1N sodium hydroxide. Upon reaching the desired pH, agitation was stopped and the whey allowed to stand for 2 minutes to permit cheese fines to settle. Three, 33 liter samples were drawn off, filtered through cheese cloth and pasteurized at 73.8, 76.7, and 79.4 C through a high-temperature short-time pastuerizer with a 25 second holding time. Flow rate through the pasteurizer was 18.9 liters per minute. A 25 ml sample of the pasteurized whey was taken 70 seconds after the first appearance of whey through the pasteurizer to insure obtaining an undiluted sample. Samples were sealed and refrigerated

at 2 C overnight prior to assaying for enzyme concentrations. After each sample was taken, the pH of the whey was adjusted to the next lower value and the procedure repeated.

Preparation of diffusion tubes

Kappa-casein was prepared according to the method of Zittle and Custer (135) substituting 100 g of purified acid casein in place of the frozen block of acid precipitated whole casein. The kappa-casein-agar substrate was prepared according to Duersch (28) and diffusion tubes were filled and stored as outlined by Holmes (55).

Measuring enzyme concentration

The procedure described by Holmes (55) was followed. Diffusion tubes were taken out of the refrigerator and allowed to equilibrate to room temperature. The wax seals were removed and 5 µl of pasteurized whey were placed on the surface of the kappa-casein-agar gel by means of a 10 µl syringe. The tubes were resealed with wax and incubated in an upright position for 48 hours at 37 C. At the end of the incubation period, diffusion distances were measured with a transmission densitometer[1] and recorded on a millivolt recorder[2]. The densitometer was fitted with a grooved tray to hold the diffusion tubes parallel to the sensor head. The chart speed of the millivolt recorder was set at 5.08 cm per minute.

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[1]Densitometer, EC 910 transmission densitometer, EC Apparatus Corp., 5000 Bark Street N., St. Petersburg, Florida 33733.

[2]Millivolt recorder, Heath Servo-recorder, model EU-208, Heath Company, Benton Harbor, Michigan 49022

Enzymes

Enzymes used in this study included a standard rennet extract[1], and *Mucor miehei* protease (MM protease)[2]. Following Ernstrom's method (36) the activity of MM protease was determined against the standard rennet extract which had an assigned activity of 100 CU/ml.

Serial dilutions of MM protease were made between 1 CU/ml and 5×10^{-4} CU/ml using standardized enzyme. A standard curve was prepared by inoculating 5 µl of each dilution into the diffusion tubes, incubating them for 48 hours at 37 C and plotting diffusion distance versus enzyme concentration on 3-cycle semilogarithmic graph paper. Enzyme concentrations of unknown samples were then read directly from the curve.

Measuring residual milk clotting activity in cheese curd

A 9 kg block of Cheddar cheese made with *Mucor miehei* protease under normal cheese making conditions was obtained from Cache Valley Dairy Association, Smithfield, Utah. Two sample plugs were taken for analysis of residual enzyme activity upon removal of the cheese from the press. The cheese block was then wrapped and stored at 4.4 C. Plug samples were pulled weekly for enzyme analysis. The holes were filled with melted paraffin to prevent mold growth, and

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[1]Obtained from Dairyland Food Laboratories, Waukesha, Wisconsin 53186

[2]Obtained from Miles Laboratories Inc., Marschall Division Madison, Wisconsin 53705

the samples were wrapped in aluminum foil to prevent moisture loss. A curd slurry was prepared by modifying Holmes' method (56). Fifteen grams of cheese curd and 200 ml of distilled water were added to a Waring blender jar. The mixture was blended at high speed for 1.5 minutes, then poured into a 300 ml glass beaker. The pH was adjusted to 6.8 by adding 1N NaOH while stirring slowly with a magnetic stirrer. The pH-adjusted slurry was allowed to stand for 25 minutes to permit settling-out of cheese fines and then the slurry was filtered through E-D filter paper[1]. Five µl portions of the filtrate were inoculated into diffusion tubes, the tubes incubated for 48 hours at 37 C, and the diffusion distances measured on the densitometer. Residual enzyme concentrations were calculated by comparing diffusion distance to the standard curve and then multiplying by a dilution factor.

In calculating the dilution factor, it was assumed that the enzyme was concentrated in the aqueous portion of the curd. The moisture content of the cheese was determined by the circulating air drying oven method (A.O.A.C., 16, 218) (93) and was found to be 38.6 percent. The dilution factor was calculated by the following formulas:

1) 15 g cheese x 38.6% H_2O in cheese = 5.8 g H_2O in cheese

2) 200 g H_2O + 5.8 g H_2O in cheese = total dilution

= 205.8 g H_2O

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[1]E-D filter paper, grade 637, 12.5 cm. The Eaton-Dikeman Company, Mount Holly Springs, Pennsylvania. 17065

Enzyme concentration was determined by plotting diffusion distance on the standard curve and reading concentration directly. The concentration was multiplied by the total dilution and divided by the number of grams of cheese to give enzyme concentration (CU) per gram cheese.

3) $$\frac{\text{CU} \times 205.8 \text{ g } H_2O}{15 \text{ g cheese}} = \frac{\text{CU}}{\text{g cheese}}$$

Enzyme dilution procedure

The measured enzyme activity of the standard *Mucor miehei* protease solution was 83 CU/ml. By adding 6 ml of the protease solute to a 100 ml volumetric flask and filling to the calibration mark with distilled water, a solution having an activity of 5 CU/ml was prepared. This procedure was repeated using 1.2 ml of protease to prepare a 1 CU/ml dilution. A 10^{-1} CU/ml dilution was prepared by pipetting 10 ml of the 1 CU/ml dilution into a 100 ml volumetric flask and filling to the calibration mark with distilled water. By pipetting 10 ml of the next highest concentrated dilution into a 100 ml volumetric flask and filling to calibration, dilutions of 10^{-2}, 10^{-3}, and 10^{-4} CU/ml were also prepared. All enzyme dilutions were prepared immediately before using and tested within one hour of dilution.

Enzyme sterilization procedure

A Millipore[1] filter assembly was dismantled and each individual part wrapped in aluminum foil prior to autoclaving for 10 minutes

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[1]Pyrex microanalysis, 25 mm, disc filter holder. Millipore Corp., Bedford, Mass. 01730

at 121.1 C. Cotton plugs were placed in the side arms of 125-ml filtering flasks. The top half of the flasks was covered with foil and the flasks autoclaved. Ten Millipore filters[1] were placed in aluminum foil and autoclaved. The stopper-fitted end of the holder base was carefully unwrapped and fitted into the flamed top of the filtering flask, leaving the end of the filter support covered. A sterile filter was placed on the filter support with flamed tongs and the sterile funnel was unwrapped and fastened on top of the filter with the spring clamp. A suction hose was attached to the filtering flask and a vacuum was applied. Starting with the least concentrated enzyme solution, the diluted samples were filtered into the flask. When the entire solution had passed through the filter, the vacuum was shut off, and the system was allowed to equilibrate (if the filter assembly was removed before the vacuum pressure was equalized, air would be sucked into the flask and contamination would occur). The filter apparatus was then removed and fitted to the flamed lip of the next filtering flask. The lip of the filtering flask containing the now-sterile enzyme solution was flamed and covered with its original foil cap. The filtering procedure was repeated for each enzyme dilution beginning with the least concentrated. The same filter was used for all dilutions.

Activity loss during filtration of the enzyme solution

Enzyme activity was measured before and after filter-sterilization

[1]GSWP 025 00, 0.22 m, 25 mm, white, plain surface. Millipore Corp., Bedford, Mass. 01730

to determine if an activity loss had occurred. No loss of activity occurred.

Test tube filling procedure

Culture tubes[1] were sterilized by autoclaving and the caps re-tightened. Sterile tubes were labeled, and placed in a test tube rack under a laminar air flow hood. Cans of "Enfamil Ready-to-Use"[2], "Enfamil Concentrated Liquid"[2], "Sustacal"[2], and "Metrecal Shape"[2] were removed from refrigeration. (Fresh samples were obtained directly from the manufacturer to eliminate the possibility of storage effects). The top of each can was sterilized immediately before opening by flooding it with 70 percent alcohol and burning the alcohol off. A flame-sterilized can opener was used to punch a hole in the top of the can through which a sterile 10 ml T.D. volumetric pipette could be inserted. The pipettes were fitted with cotton plugs prior to autoclaving. Screw caps were carefully removed from the culture tubes, one at a time, and 10 ml of the sterile milk product were pipetted into the tubes. Care was taken not to contaminate the culture tube by touching the lip or threads with the fingers and the cap was carefully replaced. The sterile pipette was neither set down between tube fillings nor wiped off. Each of the sterile milk products was pipetted into 28 culture tubes, providing 4 tubes for each enzyme dilution and 4 control tubes. A new sterile pipette was used for transferring each milk product. All transferring took place under the laminar air flow hood.

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[1]Culture tube, screw cap, Kimax Brand (Kimble 45066-A) T 1350-5, 16x150 mm. Obtained through Scientific Products (a division of American Hospital Supply Corporation) McGraw Park, Illinois 60085.

[2]Obtained from Mead Johnson Laboratories, a division of Mead Johnson and Company, Evansville, Indiana 47721.

Enzyme inoculation procedure

A 10 µl Lang-Levy pipette[1] was fitted with a 15 cm length of rubber hose, wrapped in aluminum foil, and autoclaved. The rubber hose acted as a bulb to draw solution into the pipette. By rolling the tube slowly between the thumb and forefinger, the solution was drawn up into the pipette. A constriction in the capillary of the pipette provided a self-adjustment of the volume when pressure was released on the hose. By rolling the hose between the fingers in the opposite direction, the solution was forced out of the pipette. Starting with the most dilute enzyme solution, 10 µl were pipetted onto the inside wall of each series of tubes. The lip of the culture tube was flamed quickly before replacing the cap. With the caps in place, the inoculated tubes were gently rolled in a horizontal plane to disperse the enzyme in the milk product. Liquid levels in the tubes were permanently marked so that dehydration could be observed if it occurred. The tubes were incubated in test tube racks in the dark, at 30 C and were checked weekly for signs of coagulation.

The enzyme activity of 10 µl of each dilution dispersed in 10 ml of sample was calculated to be 5×10^{-3}, 1×10^{-3}, 1×10^{-4}, 1×10^{-5}, 1×10^{-6}, and 1×10^{-7} CU/ml. Enzyme activity under normal cheese making conditions is 1.5×10^{-2} CU/ml in the cheese vat and 1.35×10^{-2} CU/ml in the whey assuming 90 percent recovery.

Bacteriological control

At the first sign of coagulation, one of the culture tubes was

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[1]Lang-Levy Pipette, 10- 1, T.D., tolerance ± 5% constriction in capillary, Scientific Products, a division of American Hospital Supply Corporation, McGraw Park, Illinois, 60085.

opened and a loopful of the sample was transferred to nutrient broth using a flamed wire loop. The broth was incubated at 30 C, and checked after 1, 2, and 5 days for turbidity or sediment formation.

Measuring progressive stages of coagulation

Progressive stages of coagulation were measured using the following values:

0 = State of the product as it comes from the can. Product flows freely.

1 = Wheying off. Whey band forming either at the bottom or the top of the milk sample.

2 = First observable thickening. Product flows slightly slower.

3 = Continued thickening. Product flows markedly slower than 2.

4 = Product flows in clumps.

5 = Product set up firm in tube or flows en masse.

The tubes were carefully tilted to allow the product to flow along the tube wall while examining for signs of thickening. The control tubes were also checked at this time for possible thickening due to storage. One of each four tubes was left untilted to measure any differences in the coagulum properties attributable to tilting.

RESULTS

Destruction of *Mucor miehei* protease in whey by HTST pasteurization

Mucor miehei protease is the most heat stable of the commercial milk clotting enzymes (27). Conditions that completely destroy MM protease activity are also severe enough to totally destroy milk clotting activity of chymosin, pepsin, EP protease, and MP protease.

Figure 1 shows the percent of original MM protease activity remaining in whey after pasteurization for 25 seconds at 73.9 C, 76.6 C, and 79.5 C and at pH values between 4.2 and 6.4. Pasteurizing at 73.9 C totally destroyed all activity at pH 6.0 and above. However, as the pH decreased heat stability of the protease at 73.9 C increased. At pH 4.2 over 95 percent of the original activity still remained after pasteurizing. The heat destruction curve for MM protease at 76.6 C parallels that for 73.9 C. *Mucor miehei* protease was completely destroyed by pastuerizing at 76.6 C at all pH values above 5.8. At pH 4.2 approximately 80 percent of the enzyme activity was still present. Pasteurizing at 79.5 completely destroyed all measurable activity at and above pH 5.4. There was little difference in enzyme stability between pH 4.6 and 4.2.

Persistence of *Mucor miehei* protease in Cheddar cheese during ripening

The persistence of *Mucor miehei* protease in ripening Cheddar cheese curd was studied over a period of 26 weeks (Table 1). During this time enzyme activity remained unchanged (Figures 9, 10, Appendix). It was hypothesized that an increase in activity would take place due to

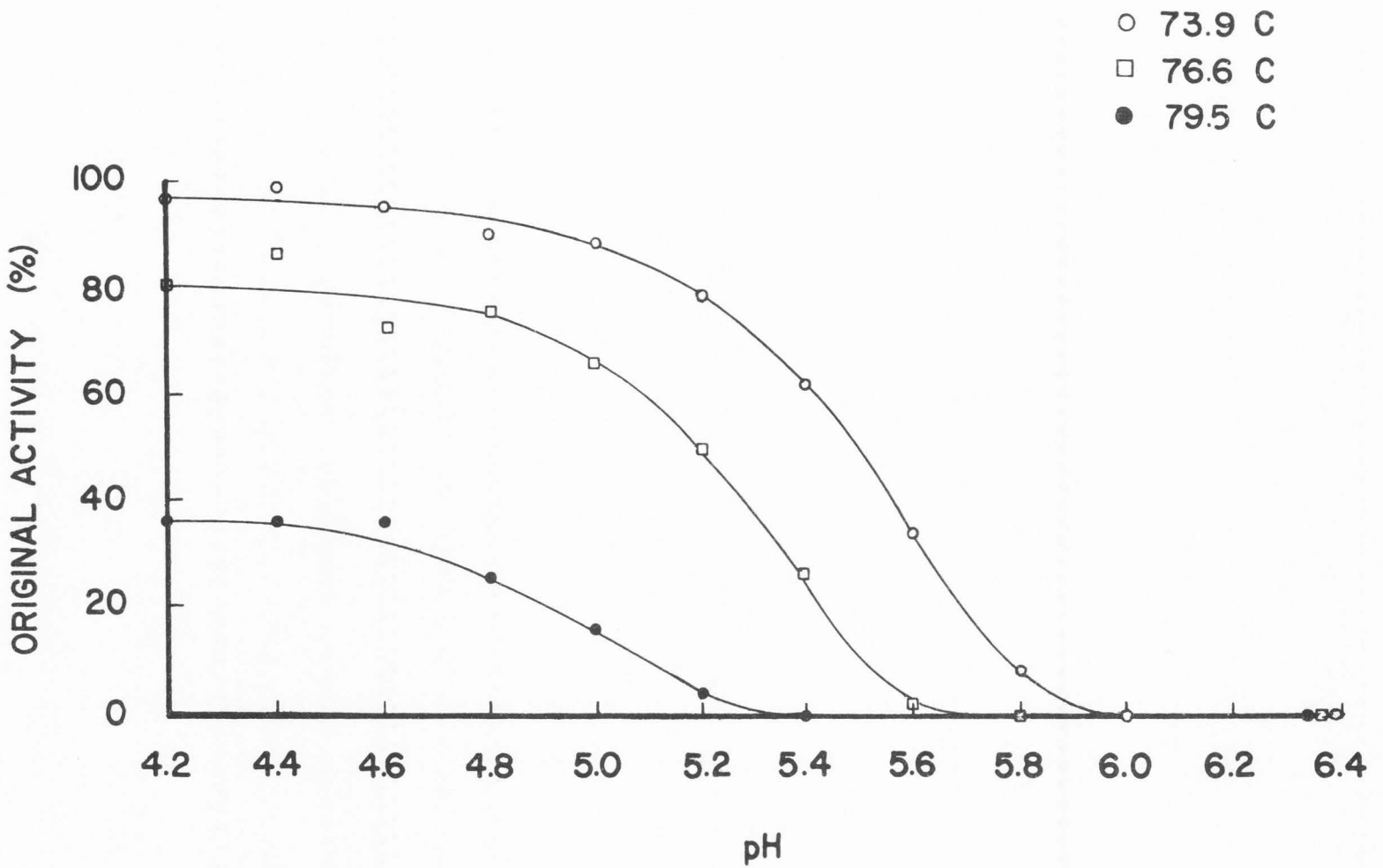


Figure 1. Destruction of *Mucor miehei* protease in whey by HTST pastuerization

Table 1
Persistence of *Mucor miehei* protease in Cheddar cheese during ripening at 4.4 C

	Cheese block I		Cheese block II	
Weeks	x 10^{-3} CU/g cheese	cv	x 10^{-3} CU/g cheese	cv
0	11.0	1.9	10.0	6.8
1	----	---	8.0	5.4
2	----	---	8.5	4.4
3	----	---	8.2	4.2
4	----	---	10.0	1.8
5	----	---	8.2	4.5
6	----	---	7.0	7.2
7	----	---	6.3	5.1
8	9.0	11.2	----	---
9	11.0	3.8	9.8	5.6
10	11.0	4.6	7.4	5.4
11	11.0	3.7	9.0	0
12	10.0	0	7.6	5.3
13	13.0	0	9.0	7.1
14	7.7	11.7	9.6	5.6
15	12.0	4.6	9.0	7.1
16	----	---	8.2	12.9
17	9.0	0	11.0	7.8
18	8.5	10.6	7.6	0
19	9.6	6.5	----	---
20	9.0	4.0	----	---
21	9.6	4.4	----	---
22	10.0	5.4	----	---
23	10.0	4.6	----	---
24	7.9	11.5	----	---
25	12.0	4.4	----	---
26	9.0	0	----	---

*Numbers represent mean values of four determinations
cv = coeffecient of variation

the release of proteolytic enzymes from lysis of starter bacteria. No such increase was observed. Fluctuations in the observed activity were due to the accuracy of the analytical method. The enzyme concentrations measured were near the lower limits of the measuring range of the linear diffusion test.

Effects of low concentrations of *Mucor miehei* on the shelf life of sterile milk products

Coagulum taken from sample tubes and inoculated into nutrient broth produced no turbidity or sediment. Coagulation of the sterile milk products was thus attributed to action of the added milk clotting enzymes and not to microbial contamination.

Concentrated infant formula. Coagulation of sterile concentrated infant formula ("Enfamil Concentrate") due to enzymatic activity of *Mucor miehei* protease was observed at concentrations as low as 1×10^{-3} CU/ml (Figure 2). Samples containing 5×10^{-3} CU/ml showed visible signs of thickening the fourth week after inoculation and a whey band after the fifth week (coagulation passed quickly through stage one to stage two with the appearance of a whey band and discernable thickening occurring almost simultaneously). After 10 weeks of storage a 2 cm whey band was present at the bottom of the tubes (Figure 3) and the product flowed in large clumps. At the end of 15 weeks the product was set up firmly but the whey band had not increased. After 20 weeks the whey band had increased to 3 cm.

Samples containing 1×10^{-3} CU/ml showed a slight whey band after 6 weeks but no thickening. After 10 weeks only the slight whey band was observable which increased to 1 cm after 15 weeks of storage. At the end of 20 weeks the whey band had not increased from 1 cm but slight thickening was observable.

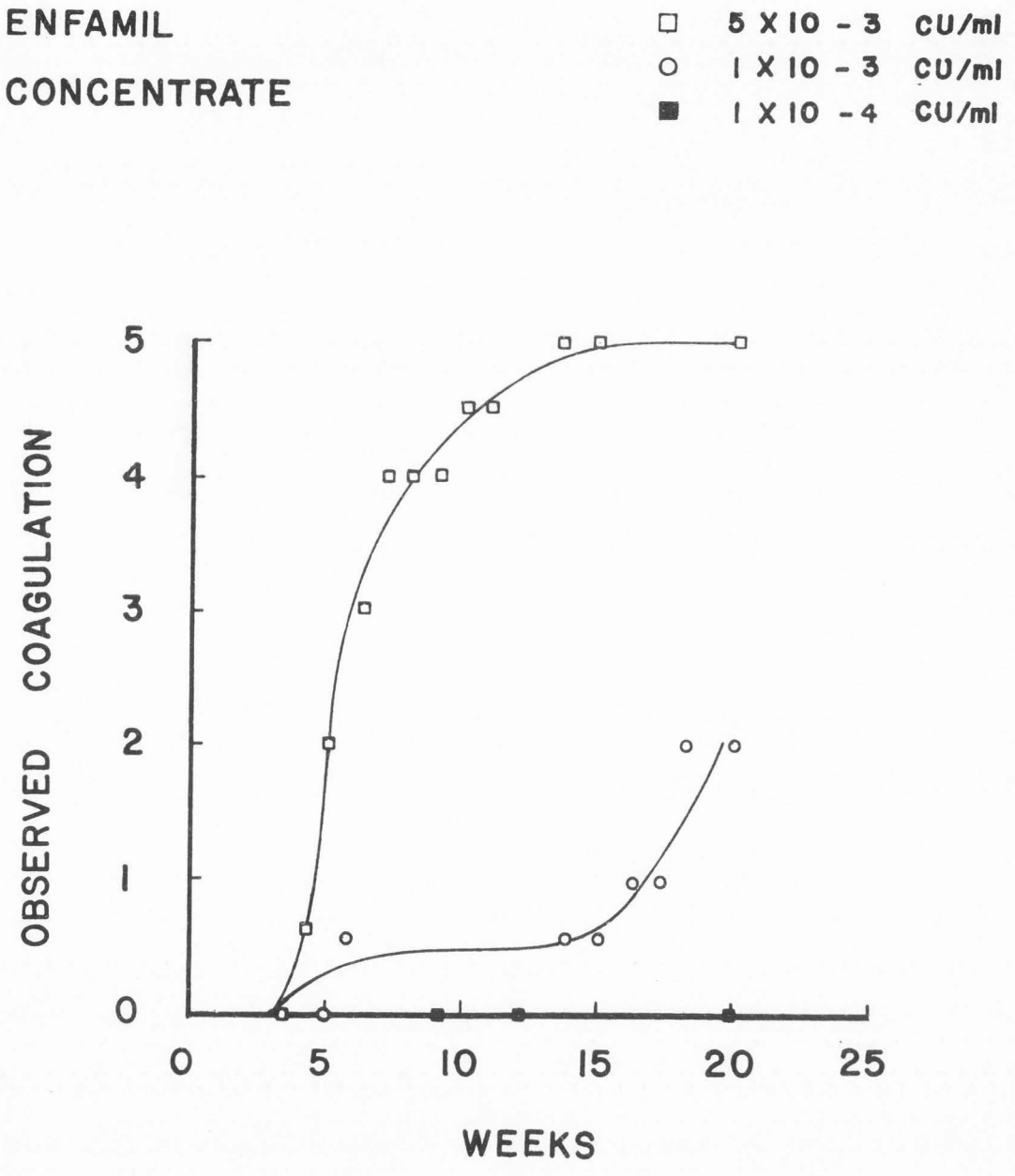
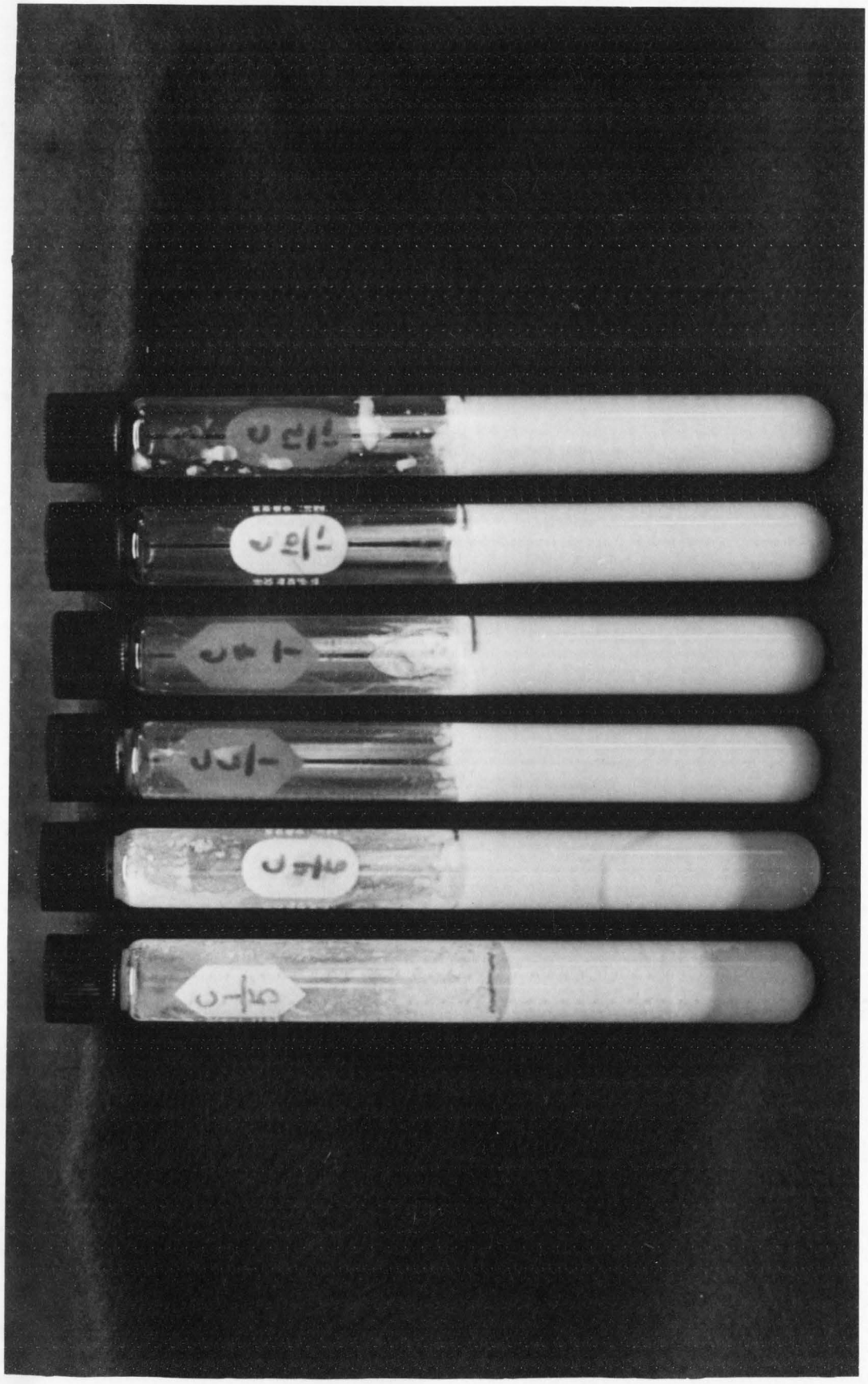


Figure 2. Coagulation of "Enfamil Concentrate" by low concentrations of *Mucor miehei* protease.

Figure 3. Tubes showing coagulation of "Enfamil Concentrate" by low concentrations of *Mucor miehei* protease. (Left to right 5×10^{-3}, 1×10^{-3}, 1×10^{-4} CU/ml.)



Diet food. Concentrations of *Mucor miehei* protease as low as 1×10^{-4} CU/ml produced coagulation of sterile diet food ("Metrecal Shape") (Figure 4). Samples containing 5×10^{-3} CU/ml showed very visible thickening after 3 weeks. As with "Enfamil Concentrate", coagulation of "Metrecal Shape" passed quickly through stage one to stage two with the whey band forming concurrently with the first signs of thickening. After 10 weeks of storage the product flowed in clumps and a 1 cm whey band was present at the top of the sample (Figure 5). At the end of 15 weeks the product was set up in the tubes with a 2 cm whey band. The whey band had not increased after 20 weeks of storage.

Samples containing 1×10^{-3} CU/ml showed slight thickening without the formation of a whey band after 3 weeks. Slight wheying off at the top of the sample was observed after 10 weeks of storage, but further thickening had not taken place. By 15 weeks the whey band had extended to 0.5 cm. Visible thickening (Stage 3-4) was observable at 17 weeks, and the whey band had extended to 1 cm. After 20 weeks the product flowed in clumps, and the whey band remained at 1 cm.

Samples with 1×10^{-4} CU/ml showed a slight wheying off after 14 weeks and definite thickening accompanied with a 0.25 cm whey band after 20 weeks.

Infant formula. The lowest concentration of *Mucor miehei* protease to produce coagulation of sterile infant formula ("Enfamil Ready-To Use") in 20 weeks was 1×10^{-4} CU/ml (Figure 6). Coagulation of "Enfamil Ready-To-Use" did not produce the same visible thickening characteristic of "Enfamil Concentrate" and "Metrecal Shape" but instead produced a very fine coagulum which was suspended in the sample liquid to give a marbled appearance. With time the fine coagulum

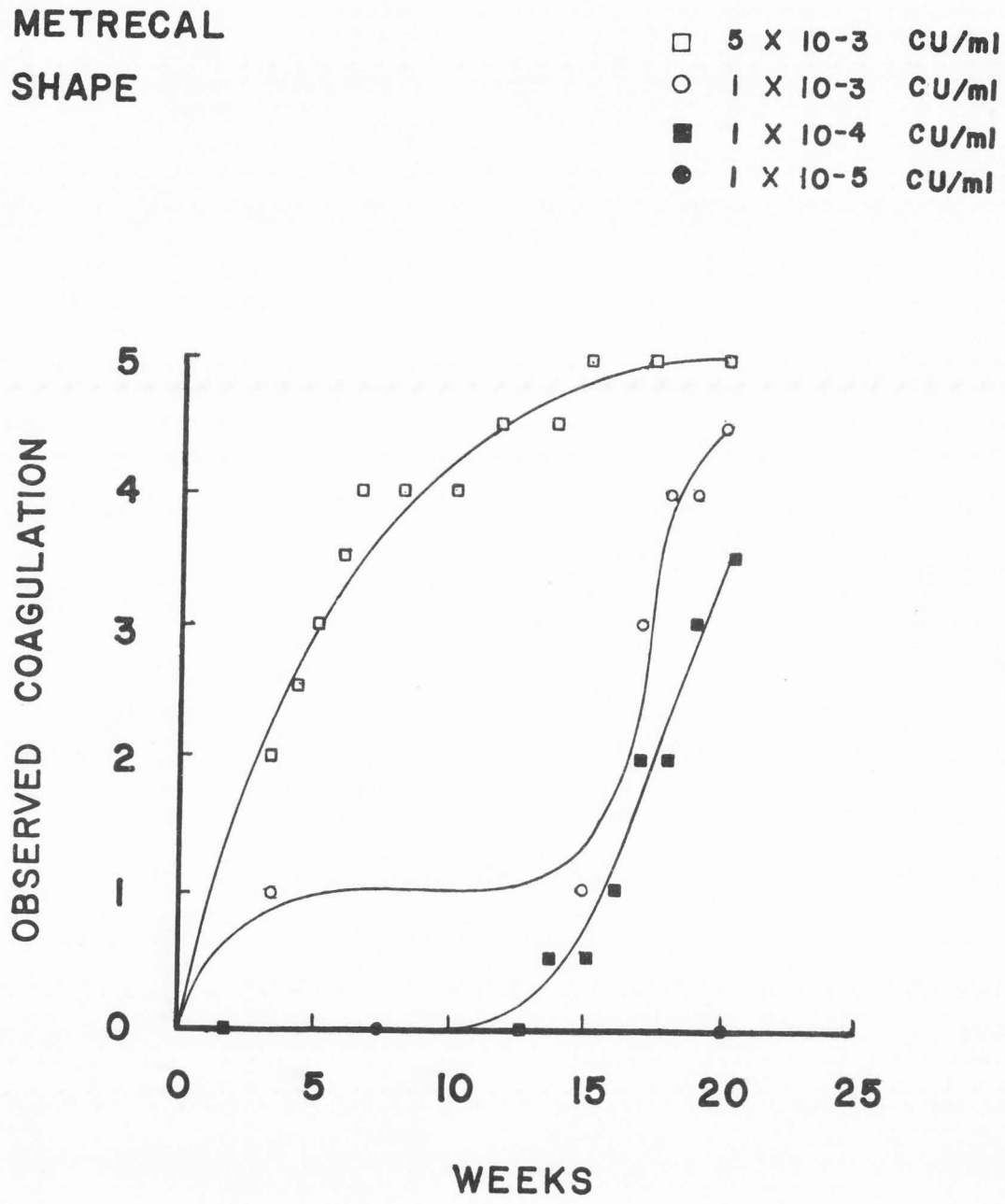


Figure 4. Coagulation of "Metrecal Shape" by low concentrations of *Mucor miehei* protease.

Figure 5. Tubes showing coagulation of "Metrecal Shape" by low concentrations of *Mucor miehei* protease. (Left to right 5×10^{-3}, 1×10^{-3}, 1×10^{-4} CU/ml, control.)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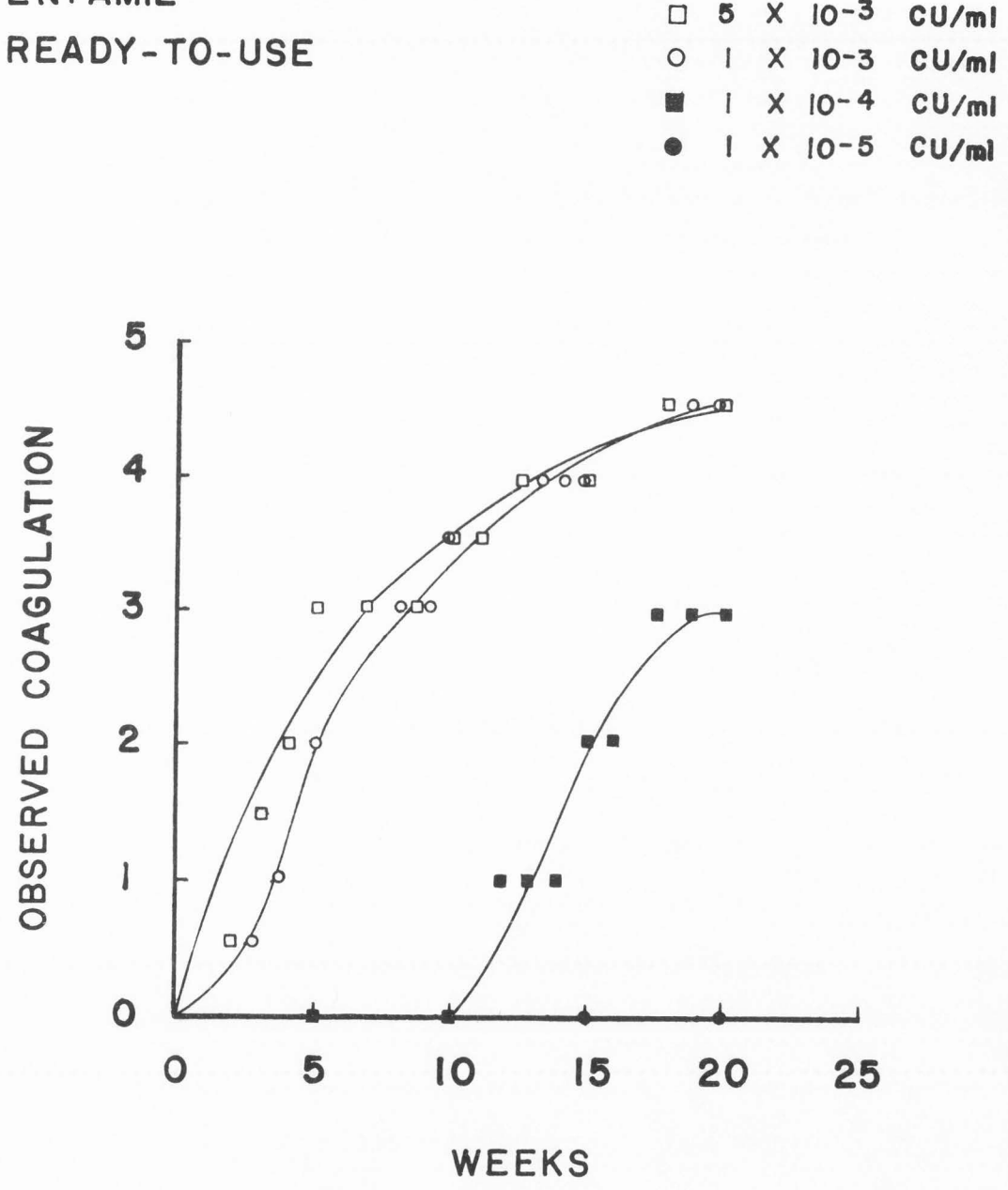


Figure 6. Coagulation of "Enfamil Ready-To-Use" by low concentrations of *Mucor miehei* protease.

loosely clumped together producing thickening and finally flowed in a loosely connected mass. This mass was quite delicate and could be easily broken apart by tilting.

Samples containing 5×10^{-3} CU/ml showed slight wheying off and very fine coagulation two weeks after inoculation. Marbling began in the third week accompanied by a 1 cm whey band and slight thickening. At the end of 10 weeks the whey band had increased to 2 cm and loose clumping of the coagulum was evident. After 15 weeks the whey band had deepened to 3 cm and the coagulum flowed in loose clumps. Figure 7 shows the extended whey band and clumping of the coagulum after 18 weeks. The whey band extended to 4 cm during 20 weeks of storage and the coagulum flowed in a loosely connected mass which was easily broken apart by movements.

Concentrations of 1×10^{-3} CU/ml produced slight wheying off after 3 weeks. Marbling of the coagulum was observable after 6 weeks accompanied by a 0.75 cm whey band. The whey band extended to 1.5 cm after 10 weeks storage and at the end of 15 weeks extended to 2 cm while the sample flowed in loosely connected clumps. After 20 weeks storage the lower half of the coagulum was clumped together into a mass while the upper half still flowed in clumps. The whey band had extended to 2.5 cm.

Samples containing 1×10^{-4} CU/ml showed no change over 11 weeks storage. After 12 weeks a 1 cm whey band started to form. This was detected by an observable band at the bottom of the tube that was less opaque than the rest of the sample. This band separated after 14 weeks into the slight green whey bands which were characteristic of

Figure 7. Tubes showing coagulation of "Enfamil Ready-To-Use" by low concentrations of *Mucor miehei* protease. (Left to right 5×10^{-3}, 1×10^{-3}, 1×10^{-4}, 1×10^{-5} CU/ml.)

those produced by higher enzyme concentrations. Marbling of the coagulum also appeared. At the end of 20 weeks the coagulum showed thickening but did not flow in clumps and the whey band extended to 2 cm.

Nutritionally complete food ("Sustacal"). After 20 weeks of storage there was no visible change in the product (figure 8).

Persistence of chymosin in Swiss cheese curd

A sample of 3-day-old, unbrined refrigerated Swiss cheese curd was analyzed for residual chymosin activity. No enzyme activity was measured.

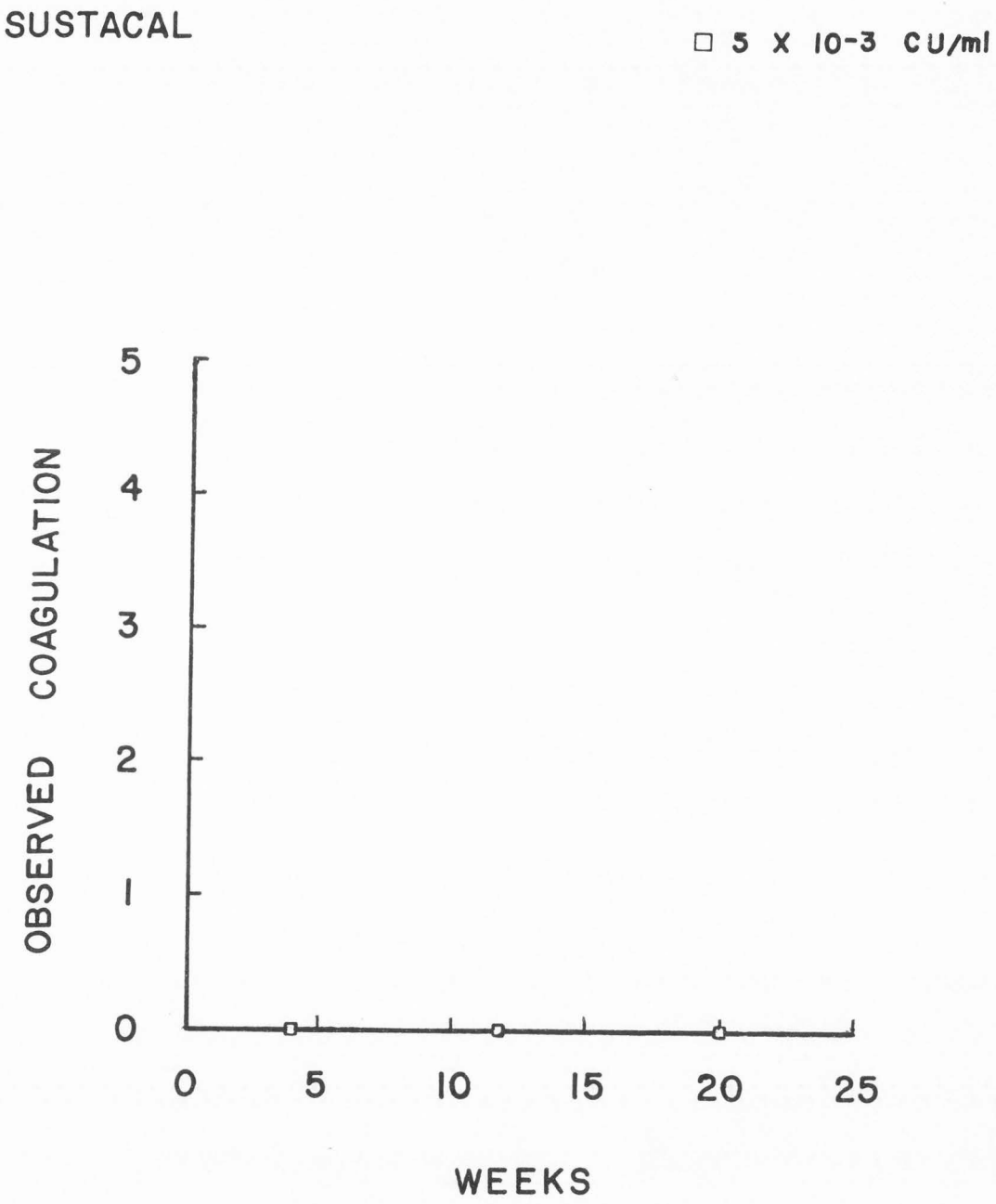


Figure 8. Coagulation of "Sustacal" by low concentrations of *Mucor miehei* protease.

DISCUSSION

The residual *Mucor miehei* protease activity in whey was destroyed by High-Temperature Short-Time pasteurization by appropriate combinations of pH and temperature. Enzyme activity in whey at pH values as low as 5.4 was completely destroyed when the whey was pasteurized at 79.5 C. When lower pasteurization temperatures were chosen the lower pH limits for complete enzyme destruction were shifted toward higher pH values. Activity in whey at pH 5.8 was destroyed at 76.6 C. At pH 6.0 and above activity was destroyed at a temperature of 73.9 C. Still lower pasteurization temperatures could be used if the pH was sufficiently high, but the flow-diversion valve on a pasteurizer is usually set at 71.8 C. thus limiting this parameter. The use of pasteurization temperatures above 79.5 C was not explored.

Because of energy costs it is more advantageous to use lower pasteurization temperatures but care must be taken to ensure that a correct pH-temperature combination is selected for enzyme activity destruction.

The persistence of *Mucor miehei* protease in ripening Cheddar cheese curd remained unchanged. This was the first attempt at measuring low enzyme concentrations in cheese curd over an extended period of time. This is an area which should receive future interest and research. Accuracy of the measurement could be improved by modifying the slurry preparation to shift measurable concentrations from the lower to the middle portion of the range of the linear diffusion test.

It is uncertain whether proteases released by the lysis of starter bacteria had any effects on the measured enzyme concentrations. The possibility exists, but until a substrate or test is devised which can differentiate between proteases at low concentrations, this will remain uncertain.

Mucor miehei protease in whey can reduce the shelf life of sterile milk products which utilize whey as an ingredient, unless enzyme activity is destroyed by the sterilization process.

"Sustacal" containing 5×10^{-3} CU/ml showed no changes after 20 weeks of storage although the product contained casein. "Enfamil Concentrate" showed signs of coagulation due to enzymatic action at 1×10^{-3} CU/ml after 6 weeks of storage. Changes produced by enzyme action were observed in "Enfamil Ready-To-Use" and "Metrecal Shape" at a concentration of 1×10^{-4} CU/ml after storage for 12 and 14 weeks respectively.

Enzyme activity in cheese milk is approximately 1.5×10^{-2} CU/ml. Raw whey used in this study contained 1.7×10^{-2} CU/ml. One liter of the whey contained 17.0 CU. Assuming 6 percent solids, the dried whey would contain 2.83×10^{-1} CU/g. "Enfamil" utilizes 7 percent lactose as an ingredient on a weight to volume basis. One gram of lactose is present in 1.43 g of dried whey. The amount of dried whey needed to provide 70 g of lactose would be 100.1 g assuming that dried whey is 70 percent lactose. The enzyme activity in 100.1 g of whey powder would be 28.3 CU. The enzyme activity per ml of "Enfamil" using 7 percent lactose as an ingredient would be 2.83×10^{-2} CU/ml. The lowest enzyme concentration which did not produce coagulation was 1×10^{-5} CU/ml. This means that 99.96 percent of the enzyme activity

present in the whey ingredient must be destroyed to prevent coagulation. Enzyme activity can be destroyed either by pasteurization prior to drying or by in-can sterilization of the milk product. Since little is known about enzyme destruction in milk during sterilization, destruction by pasteurization of the whey is recommended. Speck and Adams (114) have shown that some bacterial proteases can survive ultra-high temperature sterilization. Pasteurizing whey at pH 5.4 and above and 79.5 C will completely destroy measurable activity. Activity in whey at pH 5.8 is destroyed at 76.6 C. At pH 6.0 and above, activity is destroyed at a temperature of 73.9 C.

It should be realized that these conclusions and recommendations were based on storage of sterile milk products for 20 weeks at 30 C. If longer shelf life is desired more complete destruction of enzyme activity may be required. On the other hand, most products would be stored at lower temperatures which in turn would extend the product shelf life.

LITERATURE CITED

1. Alais, C., N. Kiger, and P. Jolles. 1967. Action of heat on cow k-casein. Heat caseino-glycopeptide. J. Dairy Sci. 50: 1738.

2. Alais, C. and G. Novak. 1970. Purification and properties of the milk coagulating enzyme derived from *Endothia parasitica*. International Dairy Congress. 1E:279.

3. Andrews, A.T. and G.C. Cheeseman. 1971. Properties of aseptically packed UHT milk: Casein modification during storage and studies with model systems. J. Dairy Res. 38:193.

4. Andrews, A.T. 1975. Properties of aseptically packed ultr-high-temperature milk. III. Formation of polymerized protein during storage at various temperatures. J. Dairy Res. 42:89.

5. Anonymous. 1972-1975. Code of Federal Regulations. Title 21. Paragraph 121.1199. Fermentation derived milk clotting enzymes.

6. Anonymous. 1960-1974. Livestock slaughter, MTAN 1-2-1. Crop Reporting Board, SRS, USDA, Washington, DC.

7. Anonymous. 1960-1975. Production of manufactured dairy products, Da 2-1. Crop Reporting Board, SRS, USDA, Washington, DC.

8. Arndt, R.H. 1972. Method of preparing a simulated milk product. U.S. Patent 3,642,493.

9. Berridge, N.J. 1952. Some observations on the determinations of the activity of rennet. Analyst 75:57.

10. Berridge, N.J. 1952. An improved method of observing the clotting of milk containing rennin. J. Dairy Res. 19:328.

11. Berridge, N.J. 1954. Rennin and the clotting of milk. In Advances in Enzymology. 15:423 E.F. Nord ed. Interscience Publishers, New York.

12. Board, P.W., J.M. Bain, and D.W. Grove. 1970. A study by electron microscopy of the structural changes occurring in whole milk during production of sterile concentrates. XVIII International Dairy Congress. IE:258.

13. Brandl, E. 1970. Differentiation of the lipolytic and proteolytic activities of various rennets and their inhibitory action on microbial lipases. XVIII International Dairy Congress. IE:284.

14. Brunner, J.R., J.W. Finley, and L. Blakely. 1969. Whey forms base for new dairy drinks. Am. Dairy Rev. 31:60.

15. Burkwall, M.P. 1973. Preparation of a high protein simulated cheese product. U.S. Patent 3,741,774.

16. Cheeseman, G.C. 1963. Action of rennet and other proteolytic enzymes on casein in casein-agar gels. J. Dairy Res. 30:17.

17. Cheeseman, G.C. 1965. Denaturation of rennin; Effect on activity and molecular configuration. Nature. 205:1011.

18. Cheeseman, G.C. and D. Knight. 1974. The nature of casein aggregates in heat and stored milk. J. Dairy Res. 41:359.

19. Christensen, V.W. 1972. Microbial coagulants in lieu of calf rennets. Am. Dairy Rev. 34:31.

20. Creamer, L.K. 1970. The action of rennin on β-casein-B. XVIII International Dairy Congress. 1E:45.

21. Creamer, L.K. and R.C. Lawrence. 1970. The action of rennets on k-casein. XVIII International Dairy Congress. 1E:44.

22. Dan, H. and N.J.T. Jespersen. 1970. Rennet-pepsin mixture for cheesemaking. XVIII International Dairy Congress. 1E:292.

23. Davidov, R.B., Y.I. Kulebjakin, and A.P. Yaroshkevich. 1970. Structural changes in proteins of sterilized milk. XVIII International Dairy Congress. 1E:261.

24. DeMan, J.M. and S.C. Batra. 1964. Measurement of the rennet clotting time of milk with an automatic clot timer. Dairy Ind. 29:32.

25. Deschamps, J.M. 1840. J. Pharm. Sci. 26:412. (Original not seen; Quoted in Methods in Enzymology Vol. 19, p. 421).

26. Dinesen, N., D.B. Emmons, D. Beckett, B. Reiser, E. Lammond, and D.M. Irvine. 1975. Cheddar cheese trials with *Mucor miehei* enzyme. J. Dairy Sci. 58:795.

27. Duersch, J.W. and C.A. Ernstrom. 1974. Heat stability of residual milk clotting enzymes in whey. J. Dairy Sci. 57:590.

28. Duersch, J.W. 1976. Heat stability of residual milk clotting enzymes in cheese whey. MS thesis. Utah State University Library, Logan, Utah. 67 p.

29. Dulley, J.R. 1974. The contribution of rennet and starter enzymes to proteolysis in cheese. Austr. J. Dairy Tech. 29:65.

30. Edelstein, D. and J.S. Jensen. 1970. Investigations on microbial rennet from *Mucor miehei*. XVIII International Dairy Congress. 1E:280.

31. Edmondson, L.F., J.K. Avants, and F.W. Douglas Jr. 1968. Utilization of whey in sterilized milk products. J. Dairy Sci. 51:931.

32. Edwards, J.L. and F.V. Kosikowski. 1969. Electrophoretic proteolytic patterns in Cheddar cheese by rennet and fungal rennets: their significance to international classification of cheese varieties. J. Dairy Sci. 52:1675.

33. Edwards, J.L. Jr. 1970. Bitterness and proteolysis in Cheddar cheese made with animal, microbial, or vegetable rennet enzymes. Diss. Abstr. Int., Sect. B. 30:4194.

34. Emmons, D.B., A. Petrasovits, R.H. Gillan, and J.M. Bain. 1970. Cheddar cheese made with rennet and pepsin. XVIII International Dairy Congress. 1E:294.

35. Emmons, D.B., B. Reiser, R. Giroux, and D. Stanley. 1974. Cheddar cheese made with bovine pepsin. XIX International Dairy Congress. 1E:721. (Original not seen; abstracted in Dairy Sci. Abstracts. 1975. V.37(5) #2350).

36. Enzyme Nomenclature 1973. Commission on Biochemical Nomenclature. Elsevier Sci. Pub. Co. Amsterdam. 449 p.

37. Ernstrom, C.A. 1958. Heterogeneity of crystalline rennin. J. Dairy Sci. 41:1663.

38. Ernstrom, C.A. 1961. Milk clotting activity of pepsin and rennin. Milk Prod. J. 52:8.

39. Ernstrom, C.A. 1961. Milk clotting enzymes and their action. In Fundamentals of Dairy Chemistry. 2nd. ed. B.H. Webb and A.H. Johnson ed. AVI Publishing Co. Inc., Westport, Connecticut.

40. Everson, T.C. and W.C. Winder. 1968. Rennet coagulation test with a recorded endpoint. J. Dairy Sci. 51:940.

41. Fed. Regist. 1972. 37:6734.

42. Foltmann, B. 1959. Sutdies on rennin. II. On the crystallization, stability, and proteolytic activity of rennin. Acta. Chem. Scand. 13:1927.

43. Foltmann, B. 1970. Prochymosin and chymosin (Prorennin and rennin). In Methods in Enzymology Vol. 19. G.E. Perlmann and L. Lorand ed. Academic Press. New York.

44. Fox, P.F. and B.F. Walley. 1971. Influence of sodium chloride on the proteolysis of casein by rennet and by pepsin. J. Dairy Res. 38:165.

45. Green, M.L. 1972. Assessment of swine, bovine and chicken pepsins as rennet substitutes for Cheddar cheese making. J. Dairy Res. 39:261.

46. Green, M.L. and A. Stackpoole. 1975. The preparation and assessment of a suitable *Mucor pusillus Lindt* protease--swine pepsin mixture for Cheddar cheese making. J. Dairy Res. 42:297.

47. Gorini, L. and G. Lanzavecchia. 1954. Recherches sur le merchanisme de production d'une proteinase bacterienne I. Nouvelle technique de determination d'une proteinase par la coagulation du lait. Biochim et. Biophys. Acta. 14:407.

48. Hamdy, A. and D. Edelstein. 1970. Some factors affecting the coagulation strength of 3 different microbial rennets. Milchwissenschaft 25:450.

49. Hankinson, C.L. 1943. The preparation of crystalline rennin. J. Dairy Sci. 26:53.

50. Hansen, K. 1970. Decomposition of casein with different milk-coagulating enzymes. XVIII International Dairy Congress. 1E:51.

51. Harper, W.J. and C.R. Lee. 1975. Residual coagulants in whey. J. Food Sci. 40:282.

52. Hindle, E.J. and J.V. Wheelock. 1969. Action of rennin on heat-sterilized milk. Biochem. J. 115:19.

53. Hindle, E.J. and J.V. Wheelock. 1970. The primary phase of rennin action in heat-sterilized milk. J. Dairy Res. 37:389.

54. Holmes, D.G. and C.A. Ernstrom. 1973. Distribution of milk-clotting enzymes between curd and whey, and their survival during Cheddar cheese making. J. Dairy Sci. 56:622.

55. Holmes, D.G. and C.A. Ernstrom. 1973. Low concentration assay of milk-clotting enzymes. J. Dairy Sci. 56:623.

56. Holmes, D.G. 1974. Distribution of milk clotting enzymes between curd and whey and their survival during Cheddar cheese manufacture. PhD Dissertation. Utah State University Library, Logan, Utah. 77 p.

57. Holsinger, V.H., L.P. Posat, E.D. Devilbiss, and M.J. Pallansch. 1973. Fortifying soft drinks with cheese whey protein. Food Tech. 27:59.

58. Hyslop, D.B., A.M. Swanson, and D.B. Lund. 1975. Heat inactivation of milk clotting enzymes. J. Dairy Sci. 58:795.

59. Itoh, T. and J. Thomason. 1971. Action of rennet and other milk clotting enzymes on casein fractions. Milchwissenschaft 26:671.

60. Kannan, A. and R. Jenness. 1961. Relation of milk serum proteins and milk salts to the effects of heat treatment on rennet clotting. J. Dairy Sci. 44:808.

61. Keay, J. 1971. Whey powder. Food Manufac. 46:36.

62. Keay, J. 1971. Experts point to expanded role for whey as a valuable ingredient in many foods. Modern Dairy. 50:24.

63. Kikuchi, T. and S. Toyoda. 1970. Use of microbial rennets in cheesemaking. XVIII International Dairy Congress. 1E:285.

64. Kisza, J., A. Druk, and A. Roskosz. 1974. Untersuchungen ueber die Hitzstabilitaet von normaler und Mastitismilch. Milchwissenschaft. 29:141.

65. Kleiner, I.S. and H. Tauber. 1932. Studies on rennin II. The isolation of prorennin. J. Biol. Chem. 96:755.

66. Klepacka, M. and E. Pijanowski. 1970. Der Einfluss von Polyphosphaten auf die Kolliodalle Stabilitaet von diskontinuierlich sterilisierter Kondensmilch. Michwissenschaft. 25:709.

67. Knight, S.G. 1966. Production of a rennin-like enzyme by molds. Can. J. Microbiol. 12:420.

68. Koning, P.D. de, P. J. van Rooijen, and A. Kok. 1969. Characterization of mixtures of calf rennet and rennet substitutes. Neth. Milk Dairy J. 23:55.

69. Koning, P.J. de, J. Koops, and P.J. van Rooijen. 1974. Some features of the heat stability of concentrated milk. II. Seasonal effects on the amounts of casein, individual whey proteins and NPN and their relation to variations in heat stability. Neth. Milk Dairy J. 28:186.

70. Kopelman, I.J. and U. Cogan. 1976. Determination of clotting power of milk clotting enzymes. J. Dairy Sci. 59:196.

71. Kopp, R., R. Michelson, D.H. Kropf, and J. Caul. 1969. Dried cottage cheese whey as a non-meat binder in an emulsion type sausage. J. Dairy Sci. 52:900.

72. Kosikowski, F.V. 1967. Greater utilization of whey powder for human consumption and nutrition. J. Dairy Sci. 50:1343.

73. Larson, M.K. and J.R. Whitaker. 1970. *Endothia parasitica* protease. Parameters affecting activity of the rennin-like enzyme. J. Dairy Sci. 53:253.

74. Larson, M.K. and J.R. Whitaker. 1970. *Endothia parasitica* protease. Parameters affecting stability of the rennin-like enzyme. J. Dairy Sci. 53:262.

75. Lawrence, R.C. and W.B. Sanderson. 1969. A micro-method for the quantitative estimation of rennets and other proteolytic enzymes. J. Dairy Res. 36:21.

76. Lea, A.S. and W.L. Dickinson. 1890. J. Physiol. 11:307.

77. Ledward, D.A., A. Finch, and W.D. Rickert. 1975. Structural and functional determinants of *M. miehei* protease. V. Enthalpy changes upon thermal denaturation in solution of varying pH. Biochim. et Biophys. Acta. 379:426.

78. Luksas, A.J. 1971. Fermenting whey and producing soy sauce from fermented whey. U.S. Patent 3, 552, 981.

79. Luksas, A.J. 1973. Preparation of a bland whey product. U.S. Patent. 3, 728, 128.

80. Martens, R. 1973. Gouda cheese made with microbial rennets derived from *Mucor miehei*. Milchwissenschaft. 28:87.

81. McBride-Warren, P.A. and W.S. Rickert. 1973. Structural and functional determinants of *Mucor miehei* protease. II. Circular dichroic studies on the native and periodate-oxidized enzyme. Biochim. et Biophy. Acta. 328:52.

82. Mickelsen, R. and C.A. Ernstrom. 1967. Factors affecting stability of rennin. J. Dairy Sci. 50:645.

83. Mickelsen, R. and N.L. Fish. 1970. Comparing proteolytic action of milk-clotting enzymes on caseins and cheese. J. Dairy Sci. 53:704.

84. Mickelsen, R. 1971. A study of rennin and pepsin stability. Diss. Abstr. Int. Sect. B. 32:3424. #71-24473.

85. Mickelsen, R. and C.A. Ernstrom. 1972. Effect of pH on the stability of rennin-porcine pepsin blends. J. Dairy Sci. 55:294.

86. Nakai, S., N.K. Wilson, and E.O. Herreid. 1964. Stability of sterile model systems of milk proteins. J. Dairy Sci. 47:1310.

87. Nakanishi, T. and T. Itoh. 1970. Effect of heat treatment on k-casein solution. XVIII International Dairy Congress. 1E:57.

88. Nelson, F.E. and W. Carson Brown. 1969. Whey utilization in fruit juice drinks. J. Dairy Sci. 52:900.

89. Nelson, J.H. 1969. Commercial scale cheese making trials with a milk clotting enzyme produced by *Mucor pusillus Lindt*. J. Dairy Sci. 52:889.

90. Nelson, J.H. 1972. Evolution in milk clotting enzymes. New opportunities and new challenges. Am. Dairy Rev. 34:37.

91. Nelson., J.H. 1975. Symposium: Application of enzyme technology to dairy manufacturing. Impact of new milk clotting enzymes on cheese technology. J. Dairy Sci. 58:1739.

92. Nielsen, V.H. 1975. Substitutes for calf rennet used in cheesemaking. Am. Dairy Rev. 37:14.

93. Association of Official Analytical Chemists. 1975. Official Methods of Analysis of the Association of Official Analytical Chemists. William Horwitz, ed. Washington D.C. 1094 p.

94. Oka, T., K. Ishino, H. Tsuzuki, K. Morihara, and K. Arima. 1973. On the specificity of a rennin-like enzyme from *Mucor pusillus*. Agri. Biol. Chem. 37:1177.

95. Oka, T. and K. Morihara. 1973. Comparative specificity of microbial acid proteinases from synthetic peptides. I. Primary specificity. Archives of Biochem. Biophys. 156:543.

96. O'Leary P.H. and P.F. Fox. 1974. A method for the quantitative analysis of the enzyme complement of commercial rennets. J. Dairy Res. 41:381.

97. Parry, R.M. Jr. 1974. Milk coagulation and protein denaturation. In Fundamentals of Dairy Chemistry. 2nd ed. B.H. Webb and A.H. Johnson ed. AVI Publishing Co. Inc., Westport, Connecticut.

98. Pelissier, J.P. and P. Manchon. 1976. Comparative study of the bitter taste of enzymatic hydrolysates from cow, ewe, and goat caseins. J. Food Sci. 41:231.

99. Phelan, J.A. 1973. Laboratory and field tests on new milk coagulants. Dairy Industries. 38:418.

100. Poznanski, S., A. Reps, J. Kowalewska, J.R. Maszewski, and L. Jedrychowski. 1974. Proteolytic activity of coagulation preparations of microbial origin depending on a kind of substrate. Milchwissenschaft. 29:742.

101. Rajagopalan, T.G., S. Moore, and W.H. Stein. 1966. Pepsin from pepsinogen, preparation and properties. J. Biol. Chem. 241.4940.

102. Rash, K.E., J.C. Colmey, and C.E. Zanzig. 1973. Confection and method of manufacture. U.S. Patent. 3,730,735.

103. Reps, A.S., S. Poznanski, and J. Jakubowski. 1975. Main properties of milk coagulating preparations of microbial origin. Milchwissenschaft. 30:65.

104. Reyes, J. 1971. A procedure for measuring residual rennin activity in whey and curd from freshly coagulated milk. MS thesis. Utah State University Library, Logan, Utah. 65 p.

105. Richardson, G.H., J.H. Nelson, R.E. Lublow, and R.L. Scharberg. 1967. Rennin-like enzyme from *Mucor pusillus* for cheese manufacture. J. Dairy Sci. 50:1066.

106. Richardson G.H. and R.V. Chaudhari. 1969. Differences between calf and adult bovine rennet extracts. J. Dairy Sci. 52:889.

107. Richardson, G.H., N.R. Gandhi, M.A. Divatia, and C.A. Ernstrom. 1971. Continuous curd tension measurements during milk coagulation. J. Dairy Sci. 54:182.

108. Samuel, R., R.W.V. Weaver, and D.B. Gammack. 1971. Changes on storage in milk processes by ultra-high-temperature sterilization J. Dairy Res. 38:323.

109. Sardinas, J.L. 1968. Rennin enzyme of *Endothia parasitica* Appli. Microbiol. 16:248.

110. Sardinas, J.L. 1972. Microbial rennets. In Advances in Applied Microbiology. 15:39. D. Perlman ed. Academic Press, New York.

111. Shovers, J. and U.S. Bavisotto. 1967. Fermentation-derived enzyme substitute for animal rennet. J. Dairy Sci. 50:942.

112. Singleton, A.D. 1973. Whey useage in dairy products. Dairy Ice Cream Field. 156:30.

113. Sommer, H.H. and H. Matsen. 1935. The relation of mastitis to rennet coagulability and curd strength of milk. J. Dairy Sci. 18:741.

114. Speck, M.L. and D.M. Adams. 1976. Heat resistant proteolytic encymes from bacterial sources. J. Dairy Sci. 59:786.

115. Sternberg, M.Z. 1971. Crystalline milk-clotting protease from *Mucor miehei* and some of its properties. J. Dairy Sci. 54:159.

116. Sternberg, M. 1972. Bond specificity, active site and milk clotting mechanism of the *Mucor miehei* protease. Biochim. et Biophys. Acta. 285:383.

117. Struble, E.R. and P.F. Sharp. 1940. Effect of heat and pH on the inactivation of rennin in whey. J. Dairy Sci. 23:587.

118. Tam, J.J. and J.R. Whitaker. 1972. Rates and extents of hydrolysis of several caseins by pepsin, rennin, *Endothia parasitica* and *Mucor pusillus* protease. J. Dairy Sci. 55:1523.

119. Tauber, H. and I.S. Kleiner. 1932. Studies on rennin I. The purification of rennin and its separation from pepsin. J. Biol. Chem. 96:745.

120. Tauber, H. and I.S. Kleiner. 1934. The chemical nature of rennin. J. Biol. Chem. 104:259.

121. Tausand, H. and W. Niclaus. 1971. Ueber eine einfache Methode zur Untersuchung von Kalberlab und Mikrobiellen Labaustauschern. Milchwissenschaft. 26:665.

122. Thompson, M.P., N.P. Tarassuk, R. Jenness, H.A. Lillevik, U.S. Ashworth, and D. Rose. 1965. Nomenclature of the proteins of cow's milk. Second revision. J. Dairy Sci. 48:159.

123. Thompson, V. 1972. Clots milk for cheese at 45% lower cost. Food Processing. 33:12.

124. Trop, M. and H. Pinsky. 1971. *Mucor pusillus* rennin as a synergist to calf rennet. J. Dairy Sci. 54:45.

125. Vajdi, M. and R.R. Pereire. 1973. The feasibility of whey utilization for the production of various drinks. Modern Dairy. 52:14.

126. Van de Beek, M.J. and S.Y. Gerlsma. 1969. Preservation of the enzymatic activity of rennin during spray drying and during storage, and the effect of sugars and certain other additives. Neth. Milk Dairy J. 23:46.

127. Vanderpoorten, R. and M. Weckx. 1972. Breakdown of casein by rennet and microbial milk-clotting enzymes. Neth. Milk Dairy J. 26:47.

128. Verhey, J.G.P. 1973. Vacuole formation in spray powder particles. III. Atomization and droplet drying. Neth. Milk Dairy J. 27:3.

129. Wang, J.T. 1969. Survival and distribution of rennin during Cheddar cheese manufacture. MS thesis. Utah State University Library, Logan, Utah. 74 p.

130. Wheelock, J.V. and E.J. Hindle. 1971. The effect of aging on heat-sterilized milk. J. Dairy Res. 38:145.

131. Wilson, H.K., J.L. Vetter, K. Sasago, and E.O. Herreid. 1963. Effects of phosphates added to concentrated milks before sterilization at ultra high temperatures. J. Dairy Sci. 46:1038.

132. Yoo, B.W. and J.F. Mattick. 1969. Utilization of acid and sweet cheese whey in wine production. J. Dairy Sci. 52:900.

133. Zadow, J.G. 1971. The influence of pH and heat-treatment on the color and stability of ultra-high-temperature sterilized milk. J. Dairy Res. 38:393.

134. Zaykovskii, I., O. Fedorova and V. Ivankin. 1928. The influence of chymosin on the proteins of milk. III. Enzymes in the stomach contents of calves at different ages. Fermentforschung 10:83. (Original not seen; abstracted in Chem. Abstr. 1929. 23:158).

135. Zittle, C.A. and J.H. Custer. 1963. Purification and some properties of α_s-casein and k-casein. J. Dairy Sci. 46:1183.

136. Zittle, C.A. 1969. Influence of heat on κ-casein. J. Dairy Sci. 52:12.

137. Zittle, C.A. 1969. Influence of heat on κ-casein. Effect of α_s-casein and concentration of calcium chloride and sodium chloride. J. Dairy Sci. 52:1356.

APPENDIX

Table 2
Percent original enzyme activity of *Mucor miehei* protease
in whey after HTST pasteurization*

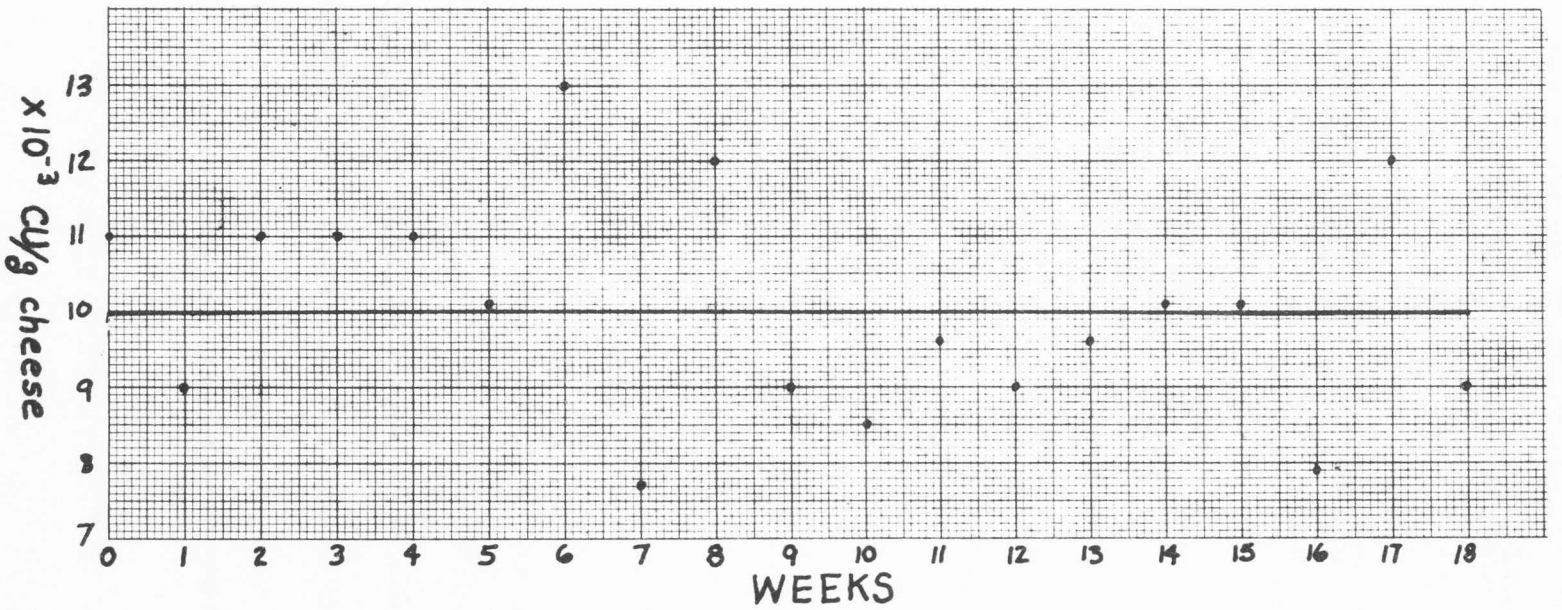
pH	Temp (F)	(C)	1	cv	2	cv	3	cv	4	cv	5	cv	$\overline{X}_5$	cv_5
6.4	165	(73.8)	0	0	0	0	0	0	0	0	0	0	0	0
	170	(76.7)	0	0	0	0	0	0	0	0	0	0	0	0
	175	(79.4)	0	0	0	0	0	0	0	0	0	0	0	0
6.2	165		0	0	0	0	0	0	0	0	0	0	0	0
	170		0	0	0	0	0	0	0	0	0	0	0	0
	175		0	0	0	0	0	0	0	0	0	0	0	0
6.0	165		0	0	0	0	0	0	0	0	0	0	0	0
	170		0	0	0	0	0	0	0	0	0	0	0	0
	175		0	0	0	0	0	0	0	0	0	0	0	0
5.8	165		20	2.8	7.5	3.8	6.4	0	7.2	3.9	6.5	6.8	9.5	5.8
	170		0	0	0	0	0	0	0	0	0	0	0	0
	175		0	0	0	0	0	0	0	0	0	0	0	0
5.6	165		49.4	2.3	33.3	2.6	35.5	2.5	13.9	10.4	32.9	2.7	30	12.6
	170		10	0	2	7.5	1.9	6.0	2.7	9.1	0	0	3.3	3.8
	175		0	0	0	0	0	0	0	0	0	0	0	0
5.4	165		49.4	7.0	80	2.2	45	2.4	61	0	76.5	4.6	62.4	15.6
	170		47	10.2	18.3	0	16.7	2.8	25	3.3	20.5	3.5	25.5	12.4
	175		0	0	0	0	0	0	0	0	0	0	0	0
5.2	165		70.6	5.7	78.3	2.2	44.8	4.7	100	0	100	2.5	78.7	23
	170		70.6	2.5	41.7	2.5	18.2	3.6	77	0	43.5	5.1	50.2	23.8
	175		10	0	2	3.1	0	0	6.1	8.3	3.4	7.9	4.3	3.8
5.0	165		88.2	2.4	100	2.1	77.3	2.1	100	6.1	76.5	2.2	88.4	11.5
	170		76.5	0	75	2.6	40.9	2.4	77.8	0	58.8	2.4	65.8	15.9
	175		35.5	0	14.2	0	5.5	4.0	20	3.0	8.8	7.2	16.8	11.8
4.8	165		---	---	91.7	1.1	72.7	2.5	100	0	97.6	3.3	90.5	12.3
	170		---	---	83	0	50	0	94.4	2.1	76.5	2.2	76	18.8
	175		38.8	0	29.2	0	5.9	4.8	34.4	2.6	17.6	7.3	25.2	13.3

*Each value in columns 1-5 represents the mean value of four determinations.

cv = coefficient of variation

Table 2 (continued)

pH	Temp (F)	(C)	1	cv	2	cv	3	cv	4	cv	5	cv	$\overline{X}_5$	cv_5
4.6	165	(73.8)	---	---	100	0	77.2	2.1	100	4.0	100	2.1	94.3	11.4
	170	(76.7)	---	---	83	0	54.5	2.3	66.6	2.7	88.2	0	73	15.4
	175	(79.4)	38.8	0	45	2.9	15.9	3.5	46.6	5.7	35.3	3.1	36.3	12.3
4.4	165		---	---	100	2.1	95.5	2.1	100	4.0	100	2.1	98.9	2.2
	170		---	---	83	3.7	77.2	1.0	94.4	2.5	94.1	2.2	87.2	8.5
	175		38.8	0	38	0	42.7	3.6	34.4	3.1	25.9	2.8	36	6.3
4.2	165		---	---	83	3.7	100	2.4	100	2.4	70.5	2.3	88.4	14.3
	170		---	---	72	2.2	77.3	1.2	94.4	2.2	76.5	2.6	80	9.8
	175		38.8	0	29.2	0	41.8	2.4	34.4	2.6	----	---	36	5.49



x = one week

y = enzyme conc.

$\Sigma_{xi} = 19$

$\Sigma_{yi} = 189.6$

$\Sigma_{xi}^2 = 19$

$\Sigma_{xiyi} = 189.6$

$$\hat{\beta}_1 = \frac{\Sigma xiyi - \Sigma xi\Sigma yi}{\Sigma x_i^2 - (\Sigma xi)^2}$$

$\hat{\beta}_1 = 9.9789$

$\hat{\beta}_o = \bar{y} - \hat{\beta}_1 \bar{x}i$

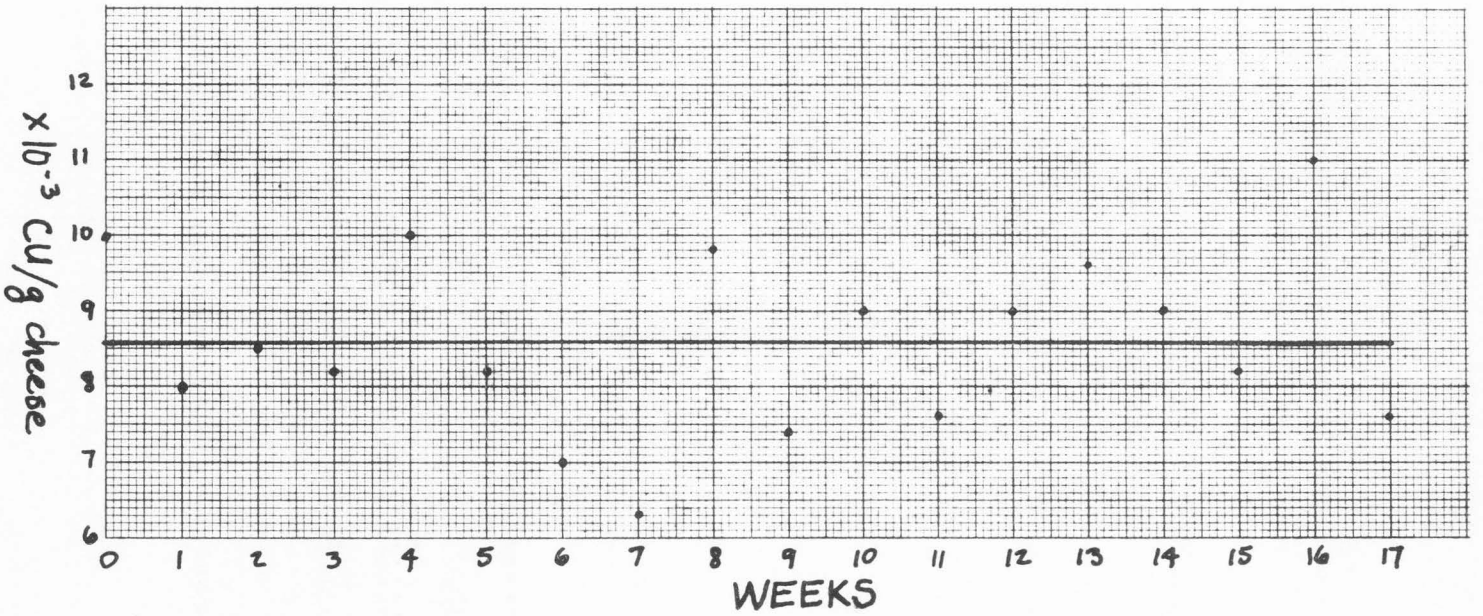
$\bar{y} = 9.9789 \quad \bar{x} = 1$

$\hat{\beta}_o = 9.9789 - 9.9789(1) = 0$

$\hat{y} = \hat{\beta}_o + \hat{\beta}_1 xi = 9.9789$

y = 9.9789 slope = 0

Figure 9. Linear regression using Least Squares Method showing persistence of *Mucor miehei* protease in Cheddar cheese during ripening at 4.4 C (Cheese block I).



x = one week

y = enzyme conc.

$\Sigma_{xi} = 18$

$\Sigma_{yi} = 154.4$

$\Sigma_{xiyi} = 154.4$

$\Sigma_{xi}^2 = 18$

$$\hat{\beta}_1 = \frac{\Sigma xiyi - \Sigma xi\Sigma yi}{\Sigma x_i^2 - (\Sigma x_i)^2}$$

$\hat{\beta}_1 = 8.5777$

$\hat{\beta}_o = \bar{y} - \hat{\beta}_1\bar{x}_i$

$\bar{y} = 8.5777 \quad \bar{x} = 1$

$\hat{\beta}_o = 8.5777 - 8.5777(1) = 0$

$\hat{y} = \hat{\beta}_o + \hat{\beta}_1 x_i = 0 + 8.5777(x)$

y = 8.5777 slope = 0

Figure 10. Linear regression using Least Squares Method showing persistence of *Mucor miehei* protease in Cheddar cheese during ripening at 4.4 C (Cheese block II).

Table 3. List of Ingredients of "Enfamil Ready-To-Use" Infant Formula and "Enfamil Concentrated Liquid" Infant Formula.

Water
Nonfat milk
Lactose
Soy and coconut oils
Soy lecithin
Carrageenan
Vitamin A palmitate
Calciferol
D-alpha-tocopheryl acetate
Sodium ascorbate
Folic acid
Thiamine hydrochloride
Riboflavin
Niacinamide
Pyridozine hydrochloride
Cyanocobalamin
Calcium pantothenate
Choline bitartrate
Ferrous sulfate
Zinc sulfate
Manganese sulfate

The products were homogenized.

Table 4. List of ingredients of "Metrecal Shape"

Concentrated sweet skim milk
Sugar
Calcium caseinate
Partially hydrogenized soy oil
Vegetable stabilizer
Artificial flavor
Carrageenan
Sodium ascorbate
Ferrous sulfate
Zinc sulfate
D-alpha tocopheryl acetate
Niacinamide
Cupric sulfate
Calcium pantothenate
Vitamin A palmitate
Pyridoxine hydrochloride
Thiamine hydrochloride
Folic acid
Biotin
Riboflavin
Calciferol
Cyanocobalamin

Table 5. List of ingredients of "Sustacal" nutritionally complete food.

Sucrose	Cyanocobalamin
Concentrated sweet skim milk	Niacinamide
Corn syrup solids	Folic acid
Partially hydrogenized soy oil	Calcium pantothenate
Sodium caseinate	Choline bitartrate
Calcium caseinate	Biotin
Soy protein isolate	Zinc sulfate
Potassium citrate	Manganese sulfate
Magnesium chloride	Supric sulfate
Artificial flavor	Sodium iodide
Dibasic magnesium phosphate	
Sodium citrate	
Calcium chloride	
Carrageenan	
Ferrous citrate	
Vitamin A palmitate	
Calciferol	
D-alpha-tocopheryl acetate	
Sodium ascorbate	
Thiamine hydrochloride	
Riboflavin	
Pyridoxine hydrochloride	

VITA

Randall Kirk Thunell

Candidate for the Degree of

Master of Science

Thesis: Persistence of *Mucor miehei* protease in Cheddar Cheese and Pasteurized Whey and Its Effect on Sterile Milk Products

Major Field: Nutrition and Food Sciences

Biographical Information:

Personal Data: Born at Logan, Utah, April 27, 1950, son of John Richard and Nedra Larsen Thunell.

Education: Attended elementary school in Logan, Utah; graduated from Sky View High School, Smithfield, Utah, in 1968; received the Bachelor of Science degree in Zoology from Utah State University in 1974.