DISTRIBUTION OF MILK CLOTTING ENZYMES BETWEEN CURD AND WHEY
AND THEIR SURVIVAL DURING CHEDDAR CHEESE MANUFACTURE

by

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DAVID HOLMES
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ABSTRACT

DISTRIBUTION OF MILK CLOTTING ENZYMES BETWEEN CURD AND WHEY
AND THEIR SURVIVAL DURING CHEDDAR CHEESE MANUFACTURE

by

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Utah State University, 1973

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A linear diffusion test in sedimentation tubes filled with casein-
agar gel successfully measured milk clotting enzymes at concentrations
of $1 \times 10^{-4}$ to $1 \times 10^{-1}$ rennin units/ml with $\pm 5\%$ accuracy. Diffusion
rates were unaffected by diluting enzyme samples with whey, 3% NaCl,
and water. The distribution of rennet, porcine pepsin, Nucor pusillus
var Lindt (MP) protease, and rennet-pepsin mixtures between curd and
whey was determined on milk coagulated at pH 5.2, 6.0, 6.4, and 6.6.
The procedure accounted for $100 \pm 7\%$ of the added enzymes. The distri-
bution of rennet was pH dependent with 31% and 72% in curd and whey re-
spectively at pH 6.6, and 86% and 17% respectively at pH 5.2. The dis-
tribution of MP protease was independent of pH with approximately 15%
and 85% in the curd and whey at all pH values. Pepsin behaved similar
to rennet but was unstable above pH 6.0. During Cheddar cheese making,
7% and 58% of the original rennet, 6% and 93% of the original MP protease,
and 5% and 17% of the original rennet-pepsin mix was active in the curd
and whey respectively at dipping. After overnight pressing, 6% of the
rennet, 3% of MP protease, and 4% of the rennet-pepsin mix remained
active in the cheese. At dipping only 9% of the original pepsin was de-
tected in the whey. Pepsin was unstable at pH values used to release
the enzyme from the curd and could not be quantitated. (77 pages)
INTRODUCTION

Rennin, a calf gastric enzyme in the form of a crude extract called rennet, has been the choice milk-clotting enzyme in commercial cheese making for many years. Because of a substantial shortage of rennet and an expanding cheese industry, cheese makers have turned to rennet substitutes to meet their needs. Some of these substitutes have undesirable effects on cheese flavor, body and texture. The reason for these effects is not clearly understood, but it is apparently due to excessive or abnormal proteolysis which results in the formation of bitter peptides (22,47,37).

Many compounds important to Cheddar cheese flavor are derived from the proteolysis of casein. However, the relative importance of milk-clotting enzymes and starter bacteria to proteolysis and flavor development during cheese curing has never been satisfactorily determined.

Good Cheddar cheese flavor as well as bitterness appears to be characteristic of particular strains of starter organisms and their response to varying conditions of cheese manufacture (22,42). Bitterness has also been attributed to the use of high rennet levels (32). Sherwood and Whitehead (71) concluded that rennet played a major role in cheese ripening, and that starter organisms were of little significance. Other studies (4,5,70,78) have also shown that cheese proteolysis was affected by the amount of rennet used to clot the milk. However, the decrease in soluble nitrogen caused by halving the normal
amount of rennet was quite small compared to the total amount developed during curing (27). Furthermore, such differences had little effect on the body or flavor of the cured cheese.

In none of the studies reported to date has it been possible to study the curing effects of starter bacteria or clotting enzymes independently. Neither has it been possible to measure the amount of clotting enzyme remaining in cheese following manufacture.

Milk-clotting enzymes added to cheese milk are partitioned between the curd and whey. It is likely that some of the activity is destroyed during cheese manufacture. If the extent of this partitioning and denaturation was more fully understood, the cheese manufacturing procedure might be altered, if necessary, to limit excessive proteolysis and bitter flavor formation attributed to specific coagulants. It may even be possible to study the curing of cheese in the absence of milk-clotting enzymes.

Conventional procedures for assaying milk-clotting enzymes are adequate for evaluating concentrated enzyme solutions, but are quite inadequate for measuring the very low concentrations found in cheese curd and whey. The purpose of this study was to develop a procedure to quantitate low levels of milk-clotting enzymes that would be present in cheese curd and whey. Then, with this test, determine the distribution of these enzymes between curd and whey and evaluate their survival during Cheddar cheese manufacture. This knowledge of the enzymes' fate could be important in evaluating rennet substitutes, and increase our understanding of the importance of clotting enzymes to the curing process.
LITERATURE REVIEW

When and how man first used milk-clotting enzymes and microorganisms for making cheese is not really known, but the importance of the type and amount of milk-clotting enzyme and type and number of microorganisms necessary to produce good quality cheese has been discussed for many years.

All proteolytic enzymes are capable of clotting milk. Some have been successfully used as replacements for rennet in cheese manufacture, but even with the introduction of these new substitutes rennin still remains the enzyme of choice and the standard against which all others are evaluated.

Cheese ripening

Before cheese is really suitable for food it must undergo a series of chemical and physical changes known as ripening. During ripening the elastic mass of concentrated milk solids is transformed into a desirable food of high nutritional value. In the early history of cheese making, it was clearly a "let alone" process in which time was the only factor considered important. As the art of cheese making developed, certain conditions were found that affected ripening. Early researchers (4,78) found that ripening was promoted by increasing the storage temperature, increasing the amount of rennet, increasing the moisture content of the cheese curd, decreasing the salt, making larger cheese, and having only a moderate acid development in the curd.
Harper and Kristofferson (36) divided cheese ripening into two general stages. The first stage included changes that occurred in the carbohydrate, fat, and protein which resulted in the accumulation of lactic acid, fatty acids, and free amino acids. The second stage involved the action of enzymes, primarily from microorganisms, on these primary compounds with the formation of secondary compounds. The relative rate and the pathways by which both primary and secondary changes proceeded, determined the type and quality of cheese.

Marth (50) grouped the components of cheese which may affect flavor into: A. Carbonyl compounds; b. Nitrogen compounds; c. Sulfur compounds; d. Fatty acids and their derivatives and; e. Miscellaneous compounds. Undoubtedly some or all of these compounds must have appeared in certain ratios if the cheese was to have a desirable flavor. But studies of chemical changes involved in the ripening or curing of Cheddar cheese have been largely concerned with protein degradation resulting from a breakdown of casein through the combined effects of milk clotting enzymes, proteolytic bacteria, and natural milk enzymes. Attempts have been made to separate the contribution of rennet toward proteolysis during cheese curing from that of lactic starters and native milk proteases.

It was first suggested by Babcock and Russell (3) that a natural enzyme in milk, which they called galactase, probably was the most important proteolytic agent in cheese ripening. Later Babcock and Russell (4) thought that since ripening progressed even during frozen storage, microorganisms played no part in ripening. They concluded that rennin was the most important ripening protease in cheese.

Van Slyke and Hart (78) demonstrated many of the conditions that increased the rate of cheese ripening, and found that most of them
favored microbial growth during the curing process. Allen (1), and Allen and Knowles (2) showed that Cheddar cheese made from milk of very low bacteria count ripened slowly, as evidenced by reduced proteolysis and slow development of flavor and texture. Davies et al (16) found that variation in the amount of pepsin and rennet appeared to have little effect on flavor, texture, and body. Their conclusion was that bacteria were the most important factors in cheese ripening.

To the contrary, Sherwood and Whitehead (71) found no specific connection between numbers of streptococci present in cheese milk at the time of renneting, and the rate of cheese ripening. They found that the proteolytic activity of various lactic starters had little influence on the rate of proteolysis in cheese. Sherwood (69) found more soluble nitrogen in rennet cheese than in pepsin cheese, but the bacterial counts were much the same. To account for greater protein degradation on the basis of bacteria, it must be assumed that the rennet cheese contained more highly proteolytic bacteria. This was not probable as the cheeses were made with the same starters and in the same manner. Therefore, the greater increase in soluble nitrogen must have been due to the rennet. Sherwood used chloroform in an attempt to eliminate the bacteria in cheese, and asses the proteolytic action of rennet during cheese ripening. His data indicated a reduction in numbers of bacteria from $10^9$ organisms/g in control cheese to $10^5$ organisms/g in chloroform-treated cheese; therefore the bacteria were only partly eliminated. In spite of this Sherwood stated, "the incorporation of chloroform (which also led to the destruction of the bacterial flora) was found to reduce markedly the extent of protein breakdown occurring in cheese, but by the addition of rennet, the diminution in breakdown was completely
restored,". He concluded that the decrease in protein breakdown was caused by a weakening of the rennet by chloroform.

In the same year, Allen and Knowles (2) published data on cheese ripening and came to the opposite conclusion, i.e., that microflora had a significant effect on proteolysis during cheese ripening.

Peterson et al (59) measured the protease activity in Cheddar cheese during making and ripening, and concluded that the active proteases responsible for ripening were largely of bacterial origin, and that only a small fraction of the total activity was contributed by the milk and rennet.

Ernstrom et al (27) showed that reducing the rennet from 90 to 45 ml/1000 pounds of milk had only a small effect on the percent soluble nitrogen in 36 week old Cheddar cheese and no effect on flavor and body scores. There was consistently more soluble nitrogen in cheese made with the higher amount of rennet, but the increase was small (approx. 3% out of 34% total soluble nitrogen). They concluded that the initial changes in normal ripening were associated with rennet action, but that the rennet was not a major factor in producing Cheddar cheese with good flavor and body.

Marth (50) published a review of research up to 1963 on the microbial and chemical aspects of cheese ripening. He revealed a similar conflicting pattern of those favoring microorganisms as opposed to rennet as the most important factor in cheese ripening. However a majority of the research supported the theory that microorganisms played the most important role.

Research at the New Zealand Dairy Research Institute (42,43,46, 47,51) has clarified the interrelated roles of starter, rennet, and
manufacturing conditions on flavor development in Cheddar cheese. They assessed relative starter activity by standardized cheese making trials and classified single strain starters into general groups. The groups were based on time from set to salt with a 2% inoculum, 0.145% titratable acidity at draining, and 0.65% titratable acidity at salting. Make-time for those in the fast group was 4 1/4 to 4 3/4 hours, and for the slow group 5 1/4 to 5 3/4 hours. Lawrence et al (42) found that cheese made with slow starters was non-bitter, and that made with the fast strains was almost invariably bitter. The strain of starter appeared to be the only factor responsible for bitterness under normal cheese making conditions. When fast strains were used, the rennet concentration affected the intensity of bitterness, but at any given rennet concentration the strain of starter determined the flavor and acceptability of the cheese. Lawrence et al (42) stated that there was little doubt that calf rennet played a role in production of bitterness in cheese, but a three fold increase over the normal amount of rennet, resulted in relatively low levels of bitterness in six-month old cheese made with slow starters. Martly and Lawrence (51) suggested that since non-bitter starters exhibited less protease activity in cheese than bitter starters, the non-bitter starters degraded high molecular weight peptides at a slow rate. They suggested that the major role of calf rennet in cheese ripening may be to degrade casein to high-molecular weight, predominantly non-bitter peptides. Increasing the rennet concentration increased the pool of such precursors, whose subsequent degradation by starter proteases led to an accumulation of low molecular-weight butter peptides. Also, since there was a relatively high amount of free amino acids in three-month
old cheese made with non-bitter starters, these starters must have had greater peptidase activity than bitter starters. The result would have been a more rapid degradation of bitter, low molecular weight peptides.

Czulak (15) had previously postulated a mechanism to explain early and transient bitterness in cheese: "the lower the pH of Cheddar cheese the greater the proteolytic activity of the rennet enzymes. The effect of the increased activity due to low pH is similar to and may be equal to that of a larger quantity of rennet at normal or higher pH values. This leads to the formation of a large pool of polypeptides, including bitter-tasting peptones. The polypeptides, including the peptones, are not broken down sufficiently to amino-acids and tend to accumulate. Hence the bitter flavor. As more and more casein is broken down, however incompletely, the increase in the free amino groups causes an upward shift in pH. This and the arrival of a secondary bacterial flora speeds up the breakdown of the polypeptides to amino-acids; the surplus of peptones is used up, and the bitter flavor gradually disappears."

A mechanism proposed by Lowrie and Lawrence (46) to account for the development of bitterness consisted of three major steps: 1. Degradation of casein by rennet to produce a pool of high molecular-weight peptides which are mostly non-bitter; 2. Hydrolysis of these peptides by starter proteases to low molecular-weight bitter peptides which; 3. May in turn be further degraded to non-bitter peptides and amino-acids by peptidases from the starter streptococci. This model differs substantially from, and challenges the validity of Czulak's model.
Stadhouders (74) pointed out that cheese containing low NaCl levels was prone to develop bitter flavors. Lawrence and Gilles (43) also showed a correlation between the amount of salt in cheese and the development of bitterness. Fox and Walley (31) suggested that the effectiveness of NaCl in controlling bitterness in Cheddar cheese may be due to its inhibitory effect on the rennin proteolysis of β-casein. They found rennin hydrolysates of β-casein were bitter but those of αs-casein were not. Proteolysis of β-casein by rennin and pepsin was completely inhibited in the presence of 10% NaCl and was reduced by 5% NaCl, while the rate of proteolysis of αs-casein was not inhibited.

Ledford et al (45) also found that β-casein was more resistant to proteolysis than αs-casein, and that the αs-casein fraction was degraded but the β-casein was largely left intact in cheese made with rennet (44).

The ultimate quality of cheese depends upon careful manufacturing and proper ripening. We have seen that cheese ripening is a complex process that involves numerous chemical, physical, and bacteriological changes. Insufficient knowledge of the specific roles of milk-clotting enzymes and microorganisms has led to a contrariety of theories of cheese ripening. The role of bacterial starters in cheese ripening and also an interrelationship of starters and rennet have been shown (42), but the role of rennet and other milk-clotting enzymes has not been well defined.

Rennin stability

The stability of rennin is affected by temperature, salts and pH (26,29,30,54). Solutions of crystalline rennin were most stable at a
pH of 5.0 to 6.0. Above pH 6.0 activity losses increased with pH and were temperature dependant. Below pH 4.5 activity losses were accompanied by an increase in the ninhydrin reaction. This suggested that activity losses were due in part to self-digestion. Chloride ions decreased rennin stability at pH 5.0 (54), and below pH 4.5 the destructive effect of chloride ions on rennin was very pronounced. Struble and Sharp (76) found that normal amounts of rennin in whey could be inactivated in 14 minutes at 50°C at pH 6.8.

The unstable characteristics of rennin above pH 6.0 suggest that during normal Cheddar cheese manufacturing, much of the rennet may be inactivated leaving little to contribute to proteolysis during cheese ripening.

Measuring enzyme activity

The usual procedure for measuring enzyme activity is to measure the rate at which products of the enzyme-catalyzed reaction appear, or the rate at which the substrate disappears. However milk-clotting is a complex process involving both a primary enzymatic phase in which κ-casein is altered and loses its ability to stabilize the remainder of the caseinate complex, and a secondary non-enzymatic phase in which aggregation of the altered caseinate takes place (23). At pH values where rennin clots milk, it does not readily hydrolyze simple peptides containing the phenylalanyl-methionine bond that is known to be hydrolyzed in κ-casein (38). Determination of the conditions necessary for the specific enzyme attack on κ-casein, and finding a simple substrate on which the primary action of milk-clotting enzymes can be followed is the object of considerable research activity (23).
The secondary phase of milk clotting is particularly susceptible to variation in milk composition. The natural variations in the composition of milk result in differences in clotting times which make it difficult to establish a standard milk substrate for enzyme assays.

Berridge (10) described a substrate made by reconstituting 12 g nonfat dry milk in 100 ml of 0.01M CaCl₂. Ten milliliters of this substrate were tempered in test tubes at 30°C in a water bath for 30 minutes and then inoculated with one milliliter of diluted rennet extract. The endpoint was taken as the first visible formation of flakes in a flowing film of milk. The flowing film was formed by dipping a stirring rod into the milk substrate, touching the rod to the side of the test tube and allowing the milk adhering to it to flow down the side of the tube. This was repeated until the end point was observed.

Sommer and Matsen (72) described an apparatus to test the clotting time of mastitic milk. The apparatus rotated 125 ml wide-mouth bottles in a water bath in an inclined position such that a film of milk was formed on the inner surface of the bottle. Fifty milliliters of the milk at 30°C were inoculated with one milliliter of a 1:50 dilution of rennet extract. Clotting time was measured by timers attached to the apparatus and the end point indicated by flake formation in the film of milk. Berridge (9) also described an automatic method of forming a flowing film of milk on the inner surface of boiling tubes by rotating the tubes in an inclined position in the water bath. Ernstrom (25) combined the Sommer-Matsen apparatus and the Berridge substrate to measure rennin activities. He found that the clotting time of the Berridge substrate continued to increase for 20 hours after its preparation when stored at 2°C. He suggested aging the sub-
strate for 20 hr at 2C before use. The activity in a rennet extract of unknown strength was measured by the time required for one milliliter of an appropriate dilution of the unknown extract to clot 25 ml of Berridge substrate. This was compared to the time required for one milliliter of a 1:250 dilution of a standard strength rennet extract to clot 25 ml of an identical substrate run at the same time in the tester. The strength of the undiluted standard was arbitrarily assigned a value of 100 rennin units (RU) per milliliter. In making such comparisons, the enzymes were diluted so they gave approximately the same clotting time. This eliminated significant errors due to deviation from the enzyme-time relationship (26). The activity in the unknown was calculated by the equation RU/ml = \( \frac{100 T_s}{T_u} \times \frac{C_s}{C_u} \)

where:

- RU/ml = rennin units/ml of unknown
- 100 = rennin units in 1 ml of undiluted standard
- \( T_s \) = clotting time of standard
- \( T_u \) = clotting time of unknown
- \( C_s \) = concentration of standard
- \( C_u \) = concentration of unknown

This method has drawbacks in that the strength of the standard rennet extract over a period of years is difficult to assess. Standard solutions are usually renewed frequently to endeavor to maintain uniform activity. Different samples of reconstituted nonfat dry milk vary in their clotting times also, so new batches must be checked against the old with an enzyme solution of known activity. The problem of standardizing the milk clotting activity of enzyme solutions has been compounded by the introduction of new non-rennin coagulants. The test has
mechanical limitations also as to the number of simultaneous experiments that can be run at one time. These problems in standardizing rennin activity make it difficult for research workers in different laboratories to compare their results.

Other assay procedures have been suggested. DeMan and Batra (17) adapted an automatic blood-clot timer for measuring rennet-clotting activity in milk. It was faster, required less substrate and had an automatic endpoint detector, but the ratio of enzyme volume to substrate volume was so high that precautions had to be taken to prevent differences in pH or salt concentrations in the enzyme solutions from affecting the clotting time.

Claesson and Nitschmann (14) used changes in optical density to continuously follow rennet coagulation. The graphs of light intensity versus time showed very clearly the beginning and progress of aggregation, formation of the gel and morphological alterations inside the aging gel. However, it was not possible to formulate the kinetics of the coagulation process by this method and find a velocity constant which was truly constant and independent of time and concentration.

Scott-Blair and Burnett (68) described a simple reproducible method for detecting the rennet-clotting time of milk based on changes in viscosity. They found that plots of changes in specific viscosity versus time of rennin action on caseinate solutions always produced the same slope. However the slope varied with different rennet extracts which they attributed to proteolytic enzymes other than rennin in the rennet extract.

Eversen and Winder (28) noted a distinct change in the ability of milk to conduct ultrasound when it clotted. The change in sound
velocity became the basis for a rennet-clotting test which gave a precise, reproducible and recorded end point.

A combination of the Sommer-Matsen apparatus and Berridge substrate appears to be the rennin activity test most generally accepted and used in rennet control laboratories.

None of the above procedures, including the Berridge-Sommer-Matsen method are sensitive enough to measure the low levels of rennin present in cheese curd and whey. Reyes (64) described a procedure for measuring residual rennin in curd and whey by using a very sensitive substrate devised by Gorini and Lanzaechia (33) and modified by Wang (79). It was approximately 12 times more sensitive than the Berridge substrate and enabled the measurement of rennin activities as low as .01 RU/ml. It was, in fact, so sensitive that salts and small amounts of casein present with rennin in the whey affected the coagulation time. Thus, for accurate results it was necessary to dilute the standard enzyme solutions with heat-treated whey or other solutions that had the same composition as the solution containing the unknown enzyme activity. Since heat treatments affect the solubility of salts in whey and also denature serum proteins, it was impossible to know that heating the whey samples did not alter its effect on coagulation of the substrate. Extreme sensitivity of the substrate also made it relatively unstable, and great care was needed to ensure standard conditions during the test.

Elliott and Emmons (20) utilized a passive indirect hemagglutination test and a corresponding inhibition test that was specific for rennin in cheese. The test was not quantitative, but could probably be made so with additional work.
Several investigators (13, 18, 65) have used radial diffusion in casein-agar gels to differentiate and characterize milk coagulants and mixtures thereof. However, the method had poor sensitivity and was not suitable for quantitative assays. Shovers et al (72) described an improved method for separating commercial milk coagulants and mixtures of coagulants by polyacrylamide gel electrophoresis. Lawrence and Sanderson (41) modified the Cheeseman procedure by the use of a thin layer of casein-agar on a defined area of a microscope slide. The rate of radial diffusion of the enzyme in the thin layer of caseinate-agar was a measure of enzyme concentration. This proved sensitive and reproducible enough to measure concentrations of proteases as low as .1 μg/ml. In our laboratory the test permitted the measurement of rennet concentrations in some cheese and whey samples, but in most instances the enzyme concentrations in cheese curd and whey were below the capabilities of the test. The procedure also required special apparatus for humidity control to prevent the agar gel from drying out during incubation. Extreme care was also needed to obtain the uniform thin layer of caseinate-agar necessary for uniform radial diffusion.

Gel diffusion techniques for the detection and quantitation of enterotoxins in foods (11, 35) have been used successfully. The concentration of enterotoxin is calculated by referring the diffusion distance to a standard curve prepared from known concentrations of enterotoxin plotted against diffusion distances. The diffusion distance is marked by a precipitate band at the antigen-antibody interface. Concentrations as low as .05 μg/ml have been detected. The antigen solution is layered over an immune-serum agar mixture in a thin bore glass tube. An
antigen-antibody precipitate is formed which progresses down the tube
as more antigen diffuses into the agar. There is a straight line re-
relationship between the distance moved by the leading edge of the band
and the square root of time (8). This conforms to Fick's law (12)
\[ \frac{\partial c}{\partial t} = D \frac{\partial^2 c}{\partial x^2} \]
where \( c \) = concentration, \( D \) = Diffusion coefficient, \( x \) =
Distance diffused, and \( t \) = Time, which on integration yields an ex-
pression for solute concentration as a function of time and distance
moved. The linear relationship means that the rate of diffusion of the
leading edge of the band is related to some fixed concentration of the
antigen as it diffuses down the tube. The density of the precipitate
at the leading edge is a function of antibody concentration only and
independent of the concentration of antigen (8).

This gel diffusion technique suggested a method for quantitating
low concentrations of milk-clotting enzymes by using a substrate
similar to that described by Lawrence and Sanderson (41).

Rennet substitutes

Many proteolytic enzymes have been suggested as replacements for
rennet in the manufacturing of cheese. Porcine pepsin was used in
the early 1900s for cheese making but it wasn't until the 1960s and
evidence of a rennet shortage that it came into major use. Since then
many researchers have recommended it as a satisfactory substitute for
part or all of the rennet (48,52,53). The research Committee of the
National Cheese Institute recommended, however, that porcine pepsin
not be used as a complete substitute for rennet (56). Because of the
instability of porcine pepsin at pH values above 6.5, slow coagulation
of milk can result in the formation of a soft curd and resultant high
fat losses (7). Ernstrom (24) found that the pepsin clotting time of skimmilk increased very rapidly from pH 6.5 to 6.7 and at a pH of 6.8, normal levels of pepsin did not clot milk. Emmons (21) suggested that at this pH pepsin is inactivated. Edelhoch (19) stated that about five or six carboxyl groups are hydrogen bonded in pepsin and the resulting network of secondary valence structures are responsible for maintaining the enzyme in its active state. Below pH 5.5 only non-hydrogen bonded carboxyl groups are dissociated and the rate of pepsin inactivation shows only a small dependence on pH. Above pH 5.5 the more stable hydrogen bonded groups tend to ionize, thus losing the secondary valence structure, therefore the rate of inactivation becomes very pH dependent. The pH range of special significance is from 6.0 to 6.7 where inactivation occurs rapidly. Green (34) suggested during Cheddar cheese manufacturing most if not all the porcine pepsin was destroyed. Wang (79) was unable to detect pepsin activity in fresh Cheddar cheese made from pepsin.

Proteolytic enzymes from plants have been tried unsuccessfully for cheese making (23,40). They are strongly proteolytic and cause bitter flavors, pasty body and reduced yields.

A microbial-derived substitute would be of value because of its essentially unrestricted availability. Literally thousands of microbes have been studied in the search for a rennet substitute. Proteases produced by bacteria have been screened, but few acceptable cheeses have been made. They generate off-flavors, render poor texture and body, and exhibit excessive proteolysis (39,62,63). For these reasons, bacterial proteases are not used commercially.
The most successful rennet substitutes have been fungal proteases. Proteases from *Endothia parasitica*, and *Mucor pusillus* var *Lindt* have been considered safe and suitable substitutes for rennet in the manufacture of cheese.

Cheddar cheese made from a protease preparation from *Endothia parasitica* (EP protease) had a bitter flavor and inferior body when compared to control cheese made with rennet (67). However, when used in making cheese where high cooking temperatures are used, the cheese was of excellent quality. It was thought that the protease was inactivated by the high cooking temperatures and therefore did not affect the cheese during curing.

A protease from *Mucor pusillus* var *Lindt* (MP protease) has been used successfully as a rennet substitute in many varieties of cheese and is now used commercially (57). Richardson et al (66) found slight bitterness in Cheddar cheese only after 14 months curing. Mickelsen and Fish (55) found MP protease less proteolytic than EP protease, but both were more proteolytic than rennin.

A protease from *Mucor miehei* (MM protease) has proved successful in cheese manufacturing also. Prins and Nielson (61) found Cheddar cheese manufactured with MM protease to be of good quality even after extended curing. Ripening, as evidenced by increased soluble nitrogen, was faster but no bitterness was evident. Sternberg (75) stated that in experimental cheese making, calf rennet and MM protease rennet were indistinguishable.
MATERIALS AND METHODS

Milk

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

Enzymes

Rennet extracted from New Zealand bobby calf vells, porcine pepsin, a protease from Mucor pusillus var Lindt, a commercial rennet-pepsin mixture, and a standard rennet extract were all obtained from Dairyland Food Laboratories, Waukesha, Wisconsin. The standard rennet extract was assigned 100 rennin units (RU) of activity per milliliter, and the activities of the other enzymes were evaluated against the standard by the method of Ernstrom (25).

Casein

Whole casein was prepared from raw skimmilk by the method of Van Slyke and Baker (77).

Preparation of test substrate

A substrate similar to that proposed by Lawrence and Sanderson (41) was modified to increase its sensitivity. The substrate contained .5% casein, 3.6% sodium acetate, .01% CaCl₂, and .7% I-on Agar.

1. Five grams of casein, 6g NaC₂H₃O₂·3H₂O and 75 ml distilled water were mixed together. The solution was stirred with a magnetic stirrer until the casein dissolved (1-3 hr.). The pH was adjusted to 5.7 by very
slow addition of .1N HCl. The solution was transferred to a 100 ml volumetric flask and made to volume with distilled water and stirred for approximately one more hour. The solution was filtered by gravity through Whatman Student Grade filter paper. The filtrate was identified as the "casein solution".

2. Six grams NaC$_2$H$_3$O$_2$·3H$_2$O, .8 g Ion Agar$^1$, 11 mg CaCl$_2$ (1 ml .1M CaCl$_2$), and 75 ml distilled water were mixed together. The pH of the solution was adjusted to 5.7 with .1N HCl and made to approximately 100 ml with distilled water. This solution was autoclaved for 10 minutes at 15 pounds pressure, cooled to 75$^\circ$C and identified as the "agar solution".

3. The casein and agar solutions were brought to approximately 75$^\circ$C in a water bath and mixed in a ratio of 10 ml casein solution to 90 ml agar solution. This mixture was identified as the "casein-agar solution".

Preparation of the diffusion tubes

Sedimentation tubes$^2$ (3 mm ID X 110 mm length) were 3/4 filled with hot casein-agar solution by means of a 10 ml syringe fitted with a plastic tube (figure 1). The tubes were then sealed with either Parafilm or paraffin wax, and stored at 2$^\circ$C.

Measurement of enzyme concentration

Tubes for the assay were removed from the refrigerator and allowed

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$^1$ Ion agar #2, Colab Laboratories (used because it gave a clearer gel than other agars).

$^2$ Tubes, Becton-Dickinson disposable sedimentation and hematocrit tube, 5 x 115 mm, #6901.
Figure 1. Filling diffusion tubes with casein-agar solution.
to come to room temperature. The wax seals were removed and 5 μl of the enzyme solution was applied with a 10 μl syringe to the top of the casein-agar gel (figure 2). Enzyme solutions of known concentration were assayed simultaneously with the unknowns. The tubes were sealed and incubated in an upright position for 48 hours at 37°C (figure 3). Diffusion distance was marked by a precipitation band formed in the casein-agar gel (figure 4). The distance from the origin to the leading edge of the precipitation band was measured with a transmission densitometer¹ and recorded on a Heathkit millivolt recorder² as shown in figure 4. A vernier caliper was also used to measure diffusion distance and it was faster, but not as precise as the densitometer. The leading edge of the precipitation band was sometimes difficult to visualize, but the densitometer clearly identified the band.

A special plastic tray was made to fit the template frame of the densitometer, such that it would hold the diffusion tubes parallel to the Sensor Head (figure 5). Grooves to hold the tubes in the plastic tray were spaced 1 cm apart for ease in placing and removing tubes.

Figure 6 shows how measurements of diffusion distance were made from the recorder tracings. A standard curve similar to figure 7, was prepared by plotting diffusion distance versus enzyme concentration on semilogarithmic graph paper. From this curve the concentration of the unknown was determined.

¹ Densitometer, EC 910 transmission densitometer, E-C Apparatus Corp., 5000 Bark Street N., St. Petersburg, Florida, 33733.

² Millivolt recorder, Heath servo-recorder, model EU-20B, Heath Company, Benton Harbor, Michigan, 49022.
Figure 2. Applying 5 ul of enzyme solution to top of casein-agar gel with a 10 ul syringe.
Figure 3. Diffusion tubes, sealed with a drop of wax and ready for incubation at 37C for 48 hours.
Figure 4. Precipitation bands formed in casein-agar gel by action of rennet as it diffused down the tube.
Figure 5. EC-910 Transmission Densitometer and Heathkit millivolt recorder set up for measuring the diffusion distance of milk-clotting enzymes in the diffusion tubes.
Figure 6. The 48 hour diffusion distance (x 1.8) of rennet as measured and drawn by the densitometer-recorder system.
Measurement of enzyme concentration in whey

A 5 µl sample of undiluted whey was applied directly to the top of the diffusion tube. Incubation, measurement of diffusion distance, and determination of concentration is explained above.

Measurement of enzyme concentration in curd

Thirty grams of curd were blended with 450 ml of distilled water at slow speed for 1 ½ min in a Waring Blender to form a curd slurry. The pH of the curd slurry was adjusted to 6.8 with 1N NaOH (63). The slurry was allowed to stand at 25°C for 30 minutes, then filtered by gravity through Whatman Student Grade filter paper. Five µl of the filtrate was applied directly to the top of the Diffusion tube for measurement of enzyme concentration.

Cheddar cheese

Cheddar cheese was made by the method of Price and Calbert (60), with slight modification. The cheese was made in 20 cm cubical plastic vats containing 7.26 kg (16 lbs) of pasteurized milk. The milk was inoculated with 2% starter at 31°C with no ripening time and set with 85 g enzyme preparation per 454 kg of milk. The cheese was cooked to 39°C. At drainage the whey was removed and measured. The curd reached pH 5.4 at milling. Pressing was accomplished in small 10 x 10 x 10 cm plastic boxes with weights placed on top. Heating was accomplished by electrical resistance. (figure 8).
Figure 8. Plastic box with electrical resistance temperature control equipment used for making Cheddar cheese.
RESULTS

Measurement of enzyme concentration

Examples of enzyme diffusion distance, as measured by the millivolt recorder, are shown in figure 9. The origin (meniscus of casein-agar gel) and the precipitation band are clearly marked. The distance from the origin to the leading edge of the precipitation band is easily measured. Figure 10 shows a curve obtained with $1 \times 10^{-1}$ RU/ml. This represents about the highest concentration of enzyme that gave good analytical response. Two bands are evident, a leading and following precipitation band. The second band was not evident at lower enzyme concentrations after 48 hours, but it appeared when the sample was left to diffuse longer (figure 11). The time needed for the second precipitation band to appear depended on the enzyme concentration. The density of the precipitate at the leading edge of the band was a function of the casein concentration and was independent of enzyme concentration. Therefore, the size and height of the curve was of no significance.

The standard curve relationship between distance diffused and the log of enzyme concentration appeared to be linear ($F>256$) (figures 12 & 13). Concentrations of rennet as low as $1 \times 10^{-4}$ RU/ml were detectable, but lower concentrations were frequently undetected and also appeared to deviate from the straight line. Thus they were considered beyond range of this test.
Figure 9. The diffusion distance (x 1.8) of rennet as measured and drawn by the densitometer-recorder system. (48 hrs.)
Figure 10. The diffusion distance measurement (x 1.8) of .1 RU/ml showing the second precipitation band (48 hrs.)
Figure 11. Charts of diffusion distance (x 1.8) measurements of rennet showing appearance of second precipitation band as diffusion proceeds.
Figure 12. Standard curve (concentration vs 48 hr. diffusion distance) of calf rennet and *Mucor pusillus* var. *Lindt* (MP) protease.
Figure 13. Standard curve (concentration vs 48 hr. diffusion distance) of a commercial rennet-pepsin mixture (R-P) and porcine pepsin.
Diffusion distances after 48 hr for several milk-clotting enzymes are given in Table 1. MP protease diffused slightly further than either rennin or pepsin. However, diffusion distances of the enzymes were generally similar. Concentrations determined from diffusion distances of known concentrations of $1 \times 10^{-4}$, $1 \times 10^{-3}$, $1 \times 10^{-1}$ RU/ml of rennet, porcine pepsin and MP protease are shown in Table 2. The average standard deviation from the known value was 5%.

In accordance with Fick's law (previously stated), the relationship between diffusion distance and the square root of time was linear (figure 14).

Effects of whey solids and salt on diffusion. Accurate measurement of rennin concentration in cheese curd and whey may be affected by substances in the whey and curd other than rennin. This was a serious problem with the sensitive clotting test used by Reyes (64). Whey was heated to 70°C for 30 minutes to inactivate the enzyme (64), then cooled and used to dilute the standard rennin solution. The rennin standard was also diluted with 0.5% and 3.0% NaCl. The effect of diluting the standard rennin with the heat-treated whey and salt solutions was compared with that of similar dilutions of the standard rennin with distilled water. Results are shown in Table 3.

The means and standard deviations showed no significant effect of salt concentrations (up to 3%), and heat-treated whey on diffusion on enzyme samples. Diffusion tubes used in this experiment are illustrated in figure 15.

Measurement of enzyme concentration in fresh curd and whey. Fresh curd and whey were prepared by the method of Reyes (64). Pasteurized whole milk was divided into four portions of 454 g each,
Table 1. Forty-eight hour diffusion distance (x 1.8) of various concentrations of rennet, porcine pepsin, Mucor pusillus var Lindt protease, and a commercial rennet-pepsin mixture.

<table>
<thead>
<tr>
<th>Concentration (RU/ml)</th>
<th>Diffusion Distance* (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rennet</td>
</tr>
<tr>
<td>1 x 10^{-1}</td>
<td>43.6 ± .8</td>
</tr>
<tr>
<td>5 x 10^{-2}</td>
<td>40.8 ± .6</td>
</tr>
<tr>
<td>2 x 10^{-2}</td>
<td>35.3 ± .5</td>
</tr>
<tr>
<td>1 x 10^{-2}</td>
<td>30.6 ± .4</td>
</tr>
<tr>
<td>5 x 10^{-3}</td>
<td>25.6 ± .5</td>
</tr>
<tr>
<td>2 x 10^{-3}</td>
<td>20.0 ± .6</td>
</tr>
<tr>
<td>1 x 10^{-3}</td>
<td>15.8 ± .7</td>
</tr>
<tr>
<td>5 x 10^{-4}</td>
<td>11.0 ± .6</td>
</tr>
<tr>
<td>2 x 10^{-4}</td>
<td>7.5 ± .5</td>
</tr>
<tr>
<td>1 x 10^{-4}</td>
<td>6.5 ± .8</td>
</tr>
</tbody>
</table>

* N = 10
Table 2. Standard deviations of measurements at four concentrations of rennet, porcine pepsin and an enzyme from Mucor pucillus var Lindt.

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>1 X 10^{-4} (s)</th>
<th>1 X 10^{-3} (s)</th>
<th>1 X 10^{-2} (s)</th>
<th>1 X 10^{-1} (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rennet</td>
<td>+ .06 X 10^{-4}</td>
<td>+ .06 X 10^{-3}</td>
<td>+ .04 X 10^{-2}</td>
<td>+ .05 X 10^{-1}</td>
</tr>
<tr>
<td>Pepsin</td>
<td>+ .06 X 10^{-4}</td>
<td>+ .05 X 10^{-3}</td>
<td>+ .04 X 10^{-2}</td>
<td>+ .06 X 10^{-1}</td>
</tr>
<tr>
<td>MP protease</td>
<td>+ .07 X 10^{-4}</td>
<td>+ .06 X 10^{-3}</td>
<td>+ .04 X 10^{-2}</td>
<td>+ .05 X 10^{-1}</td>
</tr>
</tbody>
</table>

*N = 10
Figure 14. The relationship of the diffusion distance of rennet versus the square root of diffusion time.
Table 3. Effects of diluting standard rennet extract with .5, 1.5, and 3.0% NaCl, heat treated whey, and distilled water on concentration measurements.

<table>
<thead>
<tr>
<th>Diluent</th>
<th>Concentration measurement*</th>
<th>Mean (RU/ml)</th>
<th>Standard deviation (RU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>.5% NaCl</td>
<td></td>
<td>$1.08 \times 10^{-2}$</td>
<td>$+ 0.07 \times 10^{-2}$</td>
</tr>
<tr>
<td>1.5% NaCl</td>
<td></td>
<td>$1.10 \times 10^{-2}$</td>
<td>$+ 0.08 \times 10^{-2}$</td>
</tr>
<tr>
<td>3.0% NaCl</td>
<td></td>
<td>$1.13 \times 10^{-2}$</td>
<td>$+ 0.10 \times 10^{-2}$</td>
</tr>
<tr>
<td>Heat treated whey</td>
<td></td>
<td>$1.06 \times 10^{-2}$</td>
<td>$+ 0.04 \times 10^{-2}$</td>
</tr>
<tr>
<td>Distilled water</td>
<td></td>
<td>$1.00 \times 10^{-2}$</td>
<td>$+ 0.05 \times 10^{-2}$</td>
</tr>
</tbody>
</table>

*N = 6
Figure 15. Sedimentation tubes filled with casein-agar gel showing the diffusion of rennet (1 x 10^-7 RU/ml) when diluted with salt solutions, heat treated whey and distilled water. A. diluted with distilled water; B. diluted with heat treated whey; C. diluted with .5% NaCl; and D. diluted with 3.0% NaCl.
warmed to 22°C and the pH adjusted to the desired level with 1N HCl. Four hundred fifty-four grams of each portion were warmed to 31°C in a water bath and a measured amount (equivalent to 3 oz./1000 lbs. of milk) of milk-clotting enzyme added. The samples were immediately transferred into two-225 ml centrifuge bottles and incubated at 30°C for 15 minutes past the first indications of coagulation. The curd was broken by rapid shaking, and the whey separated from the curd by centrifugation at 3500 g for 20 min in a size 1 International Centrifuge with an eight place angle head. The whey was decanted, measured and weighed. The weight and volume of curd was determined by difference. Enzyme concentration was measured in RU/ml and multiplied by the appropriate dilution factor to obtain the total amount of enzyme in the curd and whey.

**Enzyme distribution between curd and whey from freshly coagulated milk**

**Rennet distribution.** Figure 16 shows the distribution of rennet between curd and whey (Table 1, appendix). At pH 6.6 72 ± 9% of the rennet was in the whey and 31 ± 9% in the curd, but as the pH of the milk at setting was decreased, more of the rennet remained in the curd, until at the pH of 5.2, 86 ± 3% was in the curd and 17 ± 3% in the whey. Of the total rennet added to the milk, 102 ± 5% was recovered.

The pH of the milk at setting also affected the amount and formation of curd. When 454 g of milk was set at pH 5.2 the curd weighed 77 ± 8 g, was more firm, and syneresis was great. The curd weighed 122 ± 10 g and was soft with little syneresis when 454 g of milk was set at pH 6.6.
Figure 16. Distribution of rennet between curd and whey from freshly coagulated milk.
**MP protease distribution.** Figure 17 shows the distribution of MP protease between curd and whey (Table 1, appendix). At a pH of 6.6, $83 \pm 1\%$ of the enzyme was in the whey and $17 \pm 3\%$ in the curd, and changes in pH of the milk at setting had little effect on this distribution. The differences in amounts of enzyme in the curd could be accounted for by differences in weight of the curd, if the extra weight was assumed to be whey carrying the same concentration of enzyme as was in the separated whey. Of the total MP protease added to the milk, $99 \pm 2\%$ was accounted for (figure 17).

**Porcine Pepsin distribution.** The distribution of porcine pepsin between curd and whey is shown in figure 18 (Table 1, appendix). The prominent observation here was the inability to account for all the pepsin activity. The distribution between curd and whey appeared to be affected by pH in a manner similar to rennet. That is, as the pH of the milk at setting increased, the enzyme (pepsin) shifted from the curd to the whey. But at pH values above 6.5, pepsin is unstable (21,24,34), therefore, much of the activity was lost. Also in order to release the pepsin from the curd, the curd slurry had to be adjusted to pH 6.8 which caused inactivation of some of the pepsin.

**Distribution of a commercial rennet-pepsin mixture.** Figure 19 shows the distribution of a commercial rennet-pepsin mixture between curd and whey (Table 1, appendix). The effect of pH of the milk at setting on the distribution of the enzymes was similar to that of rennet, but there was a loss of total activity as the pH of the milk at setting was increased. This was attributed to the instability of pepsin.
Figure 17. Distribution of *Mucor pusillus* var. *Lindt* protease between curd and whey from freshly coagulated milk.
Figure 18. Distribution of porcine pepsin between curd and whey from freshly coagulated milk.
Figure 19. Distribution of a commercial rennet-pepsin mixture between curd and whey from freshly coagulated milk.
Distribution of a 50-50 rennin-pepsin mixture. The milk-clotting activity of a rennet solution and a porcine pepsin solution was determined by the method of Ernstrom (26). A 50% to 50% rennin-pepsin mixture (percent by activity) was prepared and used to make the curd and whey. The effect of pH of the milk at setting on the distribution of the enzyme was again similar to that of rennet (Figure 20). The distribution was also similar to the commercial rennet-pepsin mixture, however, there was a greater loss of total activity with the pure rennin-pepsin mix than with the commercial mixture.

Cheddar cheese

Measurement of enzyme concentration in curd and whey was obtained at three points during the Cheddar cheese making procedure; at cutting, at dipping, and after overnight pressing. Because of the difficulty in measuring weights of curd and whey at cutting without disrupting normal cheese making, the distribution of the enzyme in curd and whey from freshly coagulated milk was taken as representative of the distribution at cutting. Several assays of the cheese milk at this point indicated this to be true. Enzyme concentrations in the curd and in the whey were measured at dipping and measured in the curd after overnight pressing.

Distribution and survival of rennet during cheddar cheese making.

Figure 21 shows the distribution and survival of rennet at three steps of the cheese making procedure (five replications). At dipping 34 ± 4% of the rennet activity had been destroyed. The curd contained 7 ± 1% and the whey 58 ± 5% of the original activity and after overnight pressing the curd contained only 6 ± 1% of the rennet.
Figure 20. Distribution of a rennin-porcine pepsin mixture between curd and whey of freshly coagulated milk.
Figure 21. Distribution of rennet between curd and whey during Cheddar cheese making.
Distribution and survival of porcine-pepsin during cheddar cheese making. Figure 22 shows the distribution and survival of porcine pepsin during five replications of Cheddar cheese making. At dipping no activity was detected in the curd and only 9 ± 2% in the whey. After overnight pressing no activity was found in the curd. However, if small amounts of active pepsin had been in the curd they may have been destroyed by the procedure required to release the enzyme from the curd.

Distribution and survival of MP protease during cheddar cheese making. The distribution and survival of MP protease during five replications of Cheddar cheese making is shown in figure 23. At dipping, all the enzyme activity was accounted for in the curd and whey, but the curd contained only 6 ± 2% of the total enzyme added to the milk. After overnight pressing the curd contained only 3 ± 1% of the overall MP protease.

Distribution and survival of the commercial rennet-pepsin mixture during cheddar cheese making. Figure 24 shows the distribution of the activity of a commercial rennet-pepsin mixture between curd and whey of five replications of the Cheddar cheese making procedure. At dipping only 22 ± 4% of the activity was left, 5 ± 3% in the curd and 17 ± 4% in the whey. After overnight pressing, 4 ± 2% of the total activity added was left in the curd. This was presumably rennet.
Figure 22. Distribution of porcine pepsin between curd and whey during Cheddar cheese making.
Figure 23. Distribution of *Mucor pusillus* var *Lindt* protease (MP) between curd and whey during Cheddar cheese making.
Figure 24. Distribution of a commercial rennet-pepsin mix between curd and whey during Cheddar cheese making.
DISCUSSION

Detailed investigations have been carried out over many years at numerous institutions to determine the conditions and agents that are important in the development of good flavor, body and texture in cheese. Bitterness has been a problem of particular concern, especially since the introduction of rennet substitutes. Since many of the compounds that play a part in the development of cheese flavor are derived from the breakdown of casein, attempts have been made to separate the contribution of milk clotting enzymes from that of the lactic bacteria. An assay capable of measuring the low concentration of milk-clotting enzymes in cheese curd and whey was developed to assist in determining the contribution of these enzymes to cheese proteolysis.

A linear diffusion technique for quantitation of milk-clotting enzymes at very low concentration was developed and proved successful. The test compared well with and showed improved sensitivity over the micro radial-diffusion test for milk-clotting enzymes by Lawrence and Sanderson (41).

With this diffusion technique enzyme concentrations as low as $1 \times 10^{-4}$ RU/ml with a 5% standard deviation were measured. When concentrations of $10^{-1}$ RU/ml or higher were assayed, the conventional clotting test with Berridge substrate was a more precise and convenient method.
The proposed technique is approximately 60 times more sensitive than the micro radial-diffusion test of Lawrence and Sanderson (41) and requires no special apparatus for humidity control to prevent loss of moisture (just seal top of tube with wax). With the Lawrence and Sanderson method, care was needed to ensure that the enzyme solution did not flow over the edge of the center hole onto the thin caseinate agar gel during application of the sample to the test slide or during its transportation. This would cause incorrect diffusion distances. No such care was needed with the linear diffusion assay.

The linear diffusion assay was 300 times more sensitive than the clotting-time assay of Reyes (64). Whey solids and salt solutions had no significant effect on the test as they did on the clotting-time assay.

In summary, advantages and improvements of the proposed technique over other micro tests were: 1. Greater sensitivity; 2. Many tests (50-100) can be run simultaneously; 3. No special equipment is required for humidity control to prevent loss of moisture; 4. Tubes can be prepared in advance and stored in the refrigerator until needed; 5. Diffusion distance is easily measured with a densitometer; 6. Tubes may be discarded or cleaned for reuse; and 7. Whey solids and salt (up to 3%) have no significant effect on the diffusion rate of the enzymes. Therefore, activity in whey and curd slurry filtrate can be measured directly.

Some of the disadvantages encountered with the assay are: 1. It takes two days (48 hours) to complete; 2. Preparation of the casein-agar gel requires a high quality casein; and 3. Occasionally (rarely, if the substrate is prepared well) the casein-agar gel will cloud
during storage or upon removal from the refrigerator. Such tubes should be discarded. Altering the casein stability by adjusting the pH of casein solution to 5.7 too fast, cooling then warming the substrate, contaminating substrate during preparation, or a combination of the above may have caused the clouding (precipitation of casein). If more than 5-10 ul of enzyme are applied to the top of the gel, it becomes difficult to determine the origin. Therefore, the use of 5 ul is suggested in all tubes.

Differentiation of enzyme coagulants by radial diffusion on casein-agar gels (13,18,65) showed that different enzymes produced a different pattern of zones. This was also observed in this study. Figure 12 shows a second precipitation band formed by rennet. This also was observed in the differentiation tests cited above.

The diffusion rate for equal concentrations of molecules is an inverse function of molecular size, therefore, MP protease diffused slightly faster than rennin and porcine-pepsin in accordance with their molecular weights. But the differences between diffusion rates of rennet, pepsin and MP protease were small as might be expected from their molecular weights (rennin 30,000 - 34,000; pepsin 32,000 - 34,000; and MP protease 29,000 - 32,500) (23). Therefore, diffusion rates did not appear to be an effective method of qualitatively differentiating these enzymes in mixtures.

A polyacrylamide gel electrophoresis system has been developed (71) for separating commercial coagulants and mixtures of coagulants. The quantitative diffusion assay suggests an additional procedure for evaluating these enzymes. Once the coagulants have been separated and identified by gel electrophoresis, they could be eluted from the gel
and their concentrations determined by the diffusion technique. Combining these two procedures would give a qualitative and quantitative procedure for evaluating milk-clotting enzymes.

Using a sensitive milk substrate for a clotting-activity test, Heyes (64) was able to account for 91% of the rennin activity added to the milk. By the same procedure, Patel (58) was able to recover 90% of the Endothia parasitica protease. The recoverability of the linear diffusion assay was excellent, as $102 \pm 5\%$ and $99 \pm 2\%$ of the rennin and MP protease activity respectively was accounted for.

**Enzyme distribution between fresh curd and whey**

The distribution of rennet between curd and whey was profoundly affected by pH. As the pH of the milk at coagulation decreased, more rennet appeared in the curd than was released into the whey. The rennet also remained bound to the curd and was not totally measurable in curd slurries until the pH of the slurry was adjusted to 6.8.

These observations suggest that at the pH of cheese (approx. 5.2), rennet is bound to the curd by some pH dependent mechanism. If the pH at coagulation is low, more rennet will be in the cheese curd than when the pH is high.

The distribution of porcine pepsin between curd and whey was also affected by pH much the same as rennet. But because porcine pepsin is so unstable at pH values above 6.5, it was impossible to account for all of the pepsin put into the milk originally. In milk, at a pH of 6.6 - 6.8, the pepsin was partially inactivated, resulting in a loss of total recoverable activity. An even greater problem was that pepsin was bound to the curd at lower pH values, and the pH had to be raised
to 6.8 to release it. Thus, some would have been inactivated and not measured, if it had been present.

The distribution of MP protease between curd and whey, however, was not affected by pH. The pH of the milk at coagulation affected only curd formation and moisture retention in the curd. The small differences of total enzyme activity present in the curd was totally accounted for by assuming that the increase in weight of the curd at higher pH values was due to whey. This whey was assumed to contain enzyme activity equal to that in the free whey. Since pH had no effect on the binding of MP protease to the curd, no pH adjustment was necessary to release it from the curd.

The distribution of activity in a commercial rennet-pepsin mixture between fresh curd and whey was similar to that of rennin, but total recovery of activity decreased due to inactivation of the pepsin. If the commercial mixture was 50-50 as labeled and 80% of the pepsin activity was lost (distribution and survival of pepsin Figure 20), then 40% of the total activity of the mixture should have been lost. The greatest amount of activity lost was approximately 20% at pH 6.6. This suggested one of a combination of three things: 1. The rennet in the mixture lent stability to the pepsin against pH inactivation; 2. The mixture was not 50-50 rennet-pepsin under the conditions of the test; or 3. The pepsin was not porcine pepsin but bovine pepsin (or some other enzyme) which was more stable to pH inactivation.

The distribution of a pure 50-50 rennin-porcine pepsin mixture (percent by activity) between fresh curd and whey showed a greater loss of total activity than the commercial one (as much as 30% at a pH of 6.6). These observations indicated there must be some stabilizing effect
to pH inactivation of pepsin due to the mixture. It also implied that
the commercial rennet-pepsin mixture was not a 50-50 mix, or it was
not pure porcine pepsin in the mixture.

**Distribution and survival of enzymes during Cheddar cheese making**

Bitterness in cheese is generally accepted as being caused by an
accumulation of bitter peptides resulting from the breakdown of casein.
Ernstrom et al (27) found that rennet concentration had little effect
on the development of flavor in cheese, but suggested that the initial
changes in normal ripening were associated with rennet action. They
believed that characteristic cheese ripening was due more to bacterial
rather than rennet proteolysis. Lawrence et al (42) confirmed this
and further stated that good cheese flavor depended on the type and
strain of starters used and their ability to multiply under particular
cheese making conditions. They also suggested an interaction between
starters and clotting enzymes.

This work agrees with the above in that only 6.5% (.0091 RU/g)
of the total rennet added to cheese milk was left in the curd. Thus,
there was not very much to contribute to the degradation of casein.
It appeared that the important function of rennet was clot formation.
The inability to recover and measure porcine pepsin in curd made it
impossible to measure the amount of pepsin in cheese curd, but the
fact that only 9 ± 1% of the total pepsin activity remained in the
whey at dipping suggested that there was very little if any left in
the curd after pressing. Since good flavor develops in cheese made
with pepsin, and if there is little or no pepsin in the curd, flavor
development must be due to something else.
When MP protease was used to make Cheddar cheese only 3 ± 1% (.0047 RU/g) was left in the curd. Since MP protease has a greater tendency to produce bitter flavors than does rennet, two explanations may be suggested; 1. MP protease is more stable and proteolytic than rennet, and is not inactivated during the cooking process. This could permit greater proteolysis of casein during the initial stages of cheese making, increasing the pool of bitter precursors whose subsequent degradation by starter proteases may lead to the accumulation of bitter peptides; and 2. MP protease is not bound to the curd at pH 5.2 and is "free" to degrade casein more readily than is rennet. Thus bitter peptides may be formed faster until enough are accumulated to be detected. Rennet appears to be bound to the curd at the pH of cheese and may not be as free to degrade casein as fast as MP protease, therefore bitter peptide formation by rennet is slower.

These results would agree with those of Lawrence et al (41) who found that fast starters produced bitter flavors. With faster starters the pH of the cheese may decrease faster and the final pH may be lower than with slow starters and thus would bind more rennin to the curd. Stadhouders (74) also suggested that high rates of acid production during Gouda cheese manufacturing caused a greater retention of rennin in the curd. The additional rennin may therefore contribute to formation of bitter flavors early during manufacturing. It is, however, thought to be of small importance later as so little remains in the final cheese curd.

The distribution between curd and whey of Endothia parasitica protease (58) and preliminary studies on Mucor miehei protease (unpublished) showed a similar pH effect as MP protease, thus explaining in part why they cause excessive proteolysis and formation of bitter
flavors in cheese. That is, they are not bound to the curd as rennet is, and therefore may have a greater turnover number which could degrade casein gaster, causing the accumulation of bitter flavor compounds.

An alteration in cheese manufacturing such as a water wash to remove MP protease (or other rennet substitutes) may be suggested. Since it is not bound to the curd, it may wash out easily. If the cheese making procedure could be altered to remove or inactivate other rennet substitutes during manufacture, a good quality cheese, void of bitterness may be made with other rennet substitutes.
LITERATURE CITED


Table 4. Enzyme distribution* between curd and whey of freshly coagulated milk set at pH 5.2, 6.0, 6.4, and 6.6 (expressed as a percentage.)

<table>
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<th>Enzyme</th>
<th>whey</th>
<th>5.2</th>
<th>6.0</th>
<th>6.4</th>
<th>6.6</th>
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<tr>
<td>Rennet whey</td>
<td>17 + 3</td>
<td>28 + 2</td>
<td>54 + 4</td>
<td>72 + 9</td>
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<td>Rennet curd</td>
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<td>71 + 7</td>
<td>48 + 5</td>
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<tr>
<td>Total</td>
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<td>99 + 4</td>
<td>102 + 5</td>
<td>103 + 9</td>
<td></td>
</tr>
<tr>
<td>Mucor pusillus var</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>Lindt protease whey</td>
<td>90 + 1</td>
<td>83 + 2</td>
<td>82 + 2</td>
<td>83 + 1</td>
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<tr>
<td>Lindt protease curd</td>
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<td>15 + 3</td>
<td>15 + 3</td>
<td>17 + 3</td>
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<tr>
<td>Total</td>
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<td>98 + 2</td>
<td>97 + 2</td>
<td>99 + 2</td>
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<tr>
<td>Porcine pepsin whey</td>
<td>16 + 2</td>
<td>33 + 5</td>
<td>41 + 13</td>
<td>21 + 6</td>
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<td>6 + 5</td>
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<td>Total</td>
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<td>48 + 9</td>
<td>22 + 4</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>whey</td>
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<td>47 + 1</td>
<td>56 + 6</td>
<td>51 + 7</td>
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</tr>
<tr>
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<td>41 + 3</td>
<td>27 + 4</td>
<td>19 + 3</td>
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<tr>
<td>Total</td>
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<td>88 + 2</td>
<td>83 + 5</td>
<td>70 + 5</td>
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<tr>
<td>Commercial Rennet-pepsin mixture whey</td>
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<td>32 + 2</td>
<td>38 + 4</td>
<td>55 + 5</td>
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<td>50 + 3</td>
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<td>88 + 3</td>
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*N = 10