A Procedure for Measuring Residual Rennin Activity in Whey and Curd From Freshly Coagulated Milk

Jorge Reyes
Utah State University

Follow this and additional works at: https://digitalcommons.usu.edu/etd

Part of the Food Processing Commons

Recommended Citation
https://digitalcommons.usu.edu/etd/5087

This Thesis is brought to you for free and open access by the Graduate Studies at DigitalCommons@USU. It has been accepted for inclusion in All Graduate Theses and Dissertations by an authorized administrator of DigitalCommons@USU. For more information, please contact dylan.burns@usu.edu.
A PROCEDURE FOR MEASURING RESIDUAL RENNIN ACTIVITY IN WHEY AND CURD FROM FRESHLY COAGULATED MILK

by

Jorge Reyes

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

MASTER OF SCIENCE

in

Food Science and Industries

UTAH STATE UNIVERSITY
Logan, Utah

1971
ACKNOWLEDGMENT

This study was supported with funds provided by the U.S. Department of Agriculture and administered through the Utah State University Experimental Station. The Department of Food Sciences and Industries at Utah State University provided all the necessary research facilities.

I would like to express my sincere appreciation to Dr. C. A. Ernstrom for the tremendous aid and guidance he contributed to the fulfillment of this work. My appreciation is also extended to Drs. G. H. Richardson and D. Morgan for the advice and help they provided while the study was in progress. I would also like to thank Drs. C. A. Ernstrom, G. H. Richardson, D. Morgan and M. Cannon for serving as members of my graduate committee.

The tremendous encouragement by my parents, Manuel and Rosa Reyes, in pursuing an education is dearly appreciated.

This work I dedicate to my wonderful wife, DeVere, and to my two lovely children, Danny and Blair. To my wife I extend a most sincere appreciation for her support in my academic pursuits and in our home. The many hours she spent typing this manuscript will not be soon forgotten by her grateful husband.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENT</td>
<td>ii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vi</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>vii</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>REVIEW OF LITERATURE</td>
<td>3</td>
</tr>
<tr>
<td>Milk clotting enzymes</td>
<td>4</td>
</tr>
<tr>
<td>The stability of rennin</td>
<td>6</td>
</tr>
<tr>
<td>The role of residual rennin in cheese ripening</td>
<td>8</td>
</tr>
<tr>
<td>Methods for measuring rennin activity</td>
<td>10</td>
</tr>
<tr>
<td>METHODS OF PROCEDURE</td>
<td>16</td>
</tr>
<tr>
<td>Measuring rennin activity</td>
<td>16</td>
</tr>
<tr>
<td>Preparation of whey and curd for testing</td>
<td>17</td>
</tr>
<tr>
<td>Source of milk coagulants</td>
<td>19</td>
</tr>
<tr>
<td>Source of milk and subsequent treatments</td>
<td>19</td>
</tr>
<tr>
<td>RESULTS</td>
<td>20</td>
</tr>
<tr>
<td>Effect of age of substrate on coagulation time</td>
<td>20</td>
</tr>
<tr>
<td>Effect of tempering on the coagulation time of the substrate</td>
<td>20</td>
</tr>
<tr>
<td>Heat inactivation of rennet in whey at pH 5.20</td>
<td>23</td>
</tr>
<tr>
<td>Effect of diluting standard rennet extract with whey on the coagulation time of the substrate</td>
<td>26</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS (Continued)

<p>| Effect of diluting standard rennet extract with curd slurry supernatants on the coagulation time of the substrate. | 27 |
| Effect of added curd on the coagulation time of the substrate. | 29 |
| Effect of pH at setting on total rennin activity accounted for in curd and whey. | 31 |
| Effect of pH adjustments of curd slurries on recovery of rennin activity from curd. | 34 |
| Effect of dilution with water on the total rennin activity recovered from curd. | 34 |
| Effect of incubation of curd slurry on the recovery of rennin activity from curd. | 37 |
| Total rennin activities recovered from whey and curd prepared from freshly coagulated milk. | 40 |
| DISCUSSION. | 45 |
| BIBLIOGRAPHY. | 51 |</p>
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Estimate of the percentage of original rennin activity remaining in whey at pH 5.20 following 5 minute heat treatment at various temperatures</td>
<td>23</td>
</tr>
<tr>
<td>2.</td>
<td>Effect of diluting standard rennet extract with whey (pH 5.20) and water on coagulation time of substrate</td>
<td>28</td>
</tr>
<tr>
<td>3.</td>
<td>Effect of diluting standard rennet extract with curd slurry supernatants and water on the coagulation time of substrate</td>
<td>29</td>
</tr>
<tr>
<td>4.</td>
<td>Effect of added curd on the coagulation time of substrate</td>
<td>30</td>
</tr>
<tr>
<td>5.</td>
<td>Effect of incubation (25 C) of curd slurry (pH 6.80) on recovery of rennin activity from curd</td>
<td>40</td>
</tr>
<tr>
<td>6.</td>
<td>Recovery of rennin activity from curd and whey prepared from ten samples of milk (454.0 g) clotted at pH 5.20 with 6.7 RU of rennet extract</td>
<td>42</td>
</tr>
<tr>
<td>7.</td>
<td>Recovery of rennin activity from curd and whey prepared from ten samples of milk (454.0 g) clotted at pH 5.20 with 6.7 RU of crystalline rennin</td>
<td>43</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Effect of storage at 2°C on coagulation time of substrate</td>
<td>21</td>
</tr>
<tr>
<td>2.</td>
<td>Effect of tempering time on coagulation time of substrate</td>
<td>24</td>
</tr>
<tr>
<td>3.</td>
<td>Effect of pH at setting on total rennin activity recovered from whey and curd using a 1:5 dilution of curd in water</td>
<td>33</td>
</tr>
<tr>
<td>4.</td>
<td>Effect of pH on per cent of theoretical rennin activity recovered from curd diluted 1:5 in water</td>
<td>35</td>
</tr>
<tr>
<td>5.</td>
<td>Effect of curd dilution at pH 6.80 on amount of rennin activity recovered from the curd</td>
<td>38</td>
</tr>
</tbody>
</table>
ABSTRACT

A Procedure for Measuring Residual Rennin Activity in Whey and Curd from Freshly Coagulated Milk

by

Jorge Reyes, Master of Science

Utah State University, 1971

Major Professor: Dr. Carl A. Ernstrom
Department: Food Science and Industries

A procedure was developed for measuring residual rennin activity in curd extracts and whey. A sensitive substrate at pH 5.8 was prepared by mixing 6 grams of low-heat nonfat dry milk in 500 milliliters buffer containing 0.05 molar cacodylic acid, 0.02 molar calcium chloride, and 0.012 molar triethanolamine, and storing it at 2 degrees centigrade for 18 hours. Two milliliters of whey or curd extract were inoculated into 25 milliliters of substrate at 30 degrees centigrade. The coagulation time was measured and compared to that induced by 2 milliliters of a known rennin concentration added at the same time to identical substrate. Recovery of activity was determined by adjusting milk samples to pH 5.20 and coagulating them with a known concentration of rennin. The clot was broken by agitation and the curd separated from the whey by centrifugation. Activities accounted for in the curd and whey amounted to 91 ± 1.6 per cent the activity added to the milk.
Maximum release of activity from curd was achieved by diluting the curd 1:15 with water and adjusting the pH to 6.80. Inclusion of curd particles with the inoculum decreased the sensitivity of the substrate.
INTRODUCTION

Rennet has been the chief milk coagulant used in cheese manufacturing for many years. It has also been shown to proteolyze casein during cheese curing (11, 44, 52). Many other enzymes of animals (32), microbial (2, 18, 37, 40, 53) and plant (7, 26) origin also coagulate milk. A marked characteristic of most of these milk coagulants is excessive proteolysis of casein which results in bitter cheese flavors.

A shortage of rennet for cheese manufacturing has greatly increased the need for rennet substitutes. If excessive casein proteolysis could be controlled, many milk coagulants known to cause bitter flavors in cheese could be substituted for rennet. Since the extent of casein proteolysis is directly related to the amount of residual coagulant present in the cheese, a procedure capable of measuring the amount of coagulant present in the cheese at any time during and following cheese manufacture would be a tremendous aid in evaluating rennet substitutes. Knowing the fate of the coagulant may enable the cheese maker to modify the cheese making procedure in such a way as to reduce the amount of coagulant remaining in the finished cheese. Such modifications might include changes in cooking temperature, and alteration of pH at setting, cooking or dipping.
Most tests used to measure ripening of cheese, and the role of coagulants during curing are based on a measurement of non-protein nitrogen (1, 14, 44, 52). These tests are time consuming, tedious and do not reveal the amount of enzyme quantitatively. Since the amount or residual coagulant present in cheese plays a part in proteolysis during curing, a procedure that could measure enzyme activity at very low levels could be used to estimate the role of coagulants in cheese curing. It would be important that the procedure be fast and easy to perform if it were to become useful as a routine test.

The purpose of this work was to develop a procedure for testing residual levels of rennin activity in whey and curd from freshly coagulated milk. The time required for a known volume of a milk substrate to be coagulated by a known volume of enzyme solution was used as the criterion for measuring rennin activity. A sensitive substrate developed by Wang (54) was used to measure all enzyme activities.

Information obtained from this work will be used to determine the fate of milk coagulants during the cheese making process. It is hoped that the knowledge of a coagulant's fate during cheese making will aid in the future use of rennet substitutes. It is also hoped that the test procedure will help estimate the extent of proteolysis caused by residual coagulants in cheese.
"Rennin is defined as the main clotting enzyme secreted by the abomasum (fourth stomach) of suckling calves (5)." Rennet is the name given to a preparation containing the enzyme rennin along with other proteaceous materials.

The enzyme is secreted into the digestive tract of the young calf as a precursor called prorennin (29, 38). Autocatalysis of prorennin to rennin occurs at pH values lower than 5.0 and the rate increases with decreasing pH (38). Prorennin is also activated by pepsin between pH 5.5 and 6.0 (28).

Rennet has been used for many years to clot milk in manufacturing cheese. How and when man first learned to make cheese by adding the coagulant to milk is not known. Today commercial rennet is obtained by extracting rennin and prorennin from dried or fresh shredded calf vells (stomachs of calves) with solutions of sodium chloride. Prorennin is activated by adjusting the pH of the extract to approximately 4.6-5.0 until maximum activity is reached and then readjusting the pH to 5.7 (34). Preservation of rennet extract is accomplished with high concentrations of sodium chloride (14-20%) and by the addition of preservatives such as sodium benzoate, propylene glycol, ethanol, glycerol and boric acid (13).
Milk clotting enzymes. All proteolytic enzymes are capable of clotting casein in milk. Some have been suggested as replacements for rennet in manufacturing cheese.

Pepsin has been used as part or as a total substitute for rennet (12, 33). Milks clotted with this enzyme require higher titratable acidities than those clotted with rennet (30, 33). High fat losses have been found in the whey from milk clotted with pepsin (30). Since the milk clotting activity of pepsin decreases rapidly at pH values above 6.5 it seems that high fat losses in the whey of milks clotted with pepsin were due to cutting soft cheese curds formed at or near the normal pH of milk (12). Soft cheese curds tend to shatter when cut and allow trapped fat to escape from the curd to the whey (51). Protein break-down is somewhat slower in cheese made with pepsin than in rennet cheese (28, 31, 32, 44). Cheese made with mixtures of 75% pepsin and 25% rennet are equal in quality to cheese made with 100% rennet (J. B. Stine, personal correspondence).

Many microbial and vegetable proteases have been examined for possible use in cheese manufacturing. Some of these enzymes include proteases from Bacillus subtilis (23), Bacillus cereus (47), Mucor pusillus (2, 39), Endothia parasitica (40, 45), Ficus carica (26), and Withania coagulans (7). Most have been found
undesirable since they produce bitterness in cheese (23, 24).

Cheese made with plant proteases develop bitterness in the early stages of ripening, but some cheese coagulated with proteases derived from *Withania coagulans* (7) and *Ficus carica* (26) occasionally become less bitter with continued ripening.

Many fungi and bacteria produce enzymes that clot milk, but only a few of these enzymes produce cheese of desirable quality.

Richardson *et al.* (39) found the characteristics of a protease from *Mucor pusillus var. Lindt* to be similar to those of rennet. They showed that proteolytic activities on casein, clotting time of identical substrates at various pH values, curd tensions of milk clotted as a function of time and curd tensions of milk clotted as a function of temperature were much alike for the fungal protease and rennet. Mild Cheddar, Brick, Parmesan and Pasta Filata cheeses of good quality were prepared with the new protease. However, a bitter flavor developed in the Cheddar cheese after 14 months ripening.

Babel and Somkuti (2) obtained Cheddar cheese that was critized for rancid flavor and a coarse mealy body when a protease from *Mucor pusillus* was used. The difference between their cheese and that made by Richardson *et al.* was probably due to the variety of organism from which the protease was derived.
Irvin, Puhan, and Gruetzner (23) reported that excellent Cheddar cheese was made with a protease produced by a mutated strain of *Bacillus subtilis*. Sardinas (40) patented a protease from *Endothia parasitica* that can be substituted for rennet. Cheddar, Swiss, Monterey, Colby, Muenster, Limburger, Mozzarella, Provolone, Romano, and Asiago cheese of quality equal to or superior to rennet cheese was reported by Shovers and Bavisotto (45) to have been made with the protease from *Endothia parasitica*. However, Cheddar cheese made with the protease from *Endothia parasitica* developed bitterness during curing (C. A. Ernstrom and J. Reyes, unpublished data). Swiss cheese made with the same protease did not develop bitter flavors (C. A. Ernstrom, personal communication). The cooking temperatures used in making Cheddar (39-40 °C) and Swiss (53-54 °C) cheese was probably responsible for destruction of much of the enzyme in Swiss cheese, but not in Cheddar cheese.

**The stability of rennin.** Any procedure for measuring rennin activity must be concerned with stability of the enzyme. Stability is affected by pH, temperature, ionic strength and specific ions in the substrate (34). It is also important to know the effect of light, agitation, and various chemicals on the stability of the enzyme. An effective test procedure should minimize or eliminate in the substrate all factors contributing to instability
of the enzyme and amplify as many favorable factors as possible.

The milk clotting activity of rennin is known to be affected by heat (16, 36), pH (17, 34), ultrasonic vibrations (13), shaking (43), visible and ultra violet light (13), and by antiseptics such as chloroform and formaldehyde (8, 44).

Foltman showed that solutions of crystalline rennin were most stable at pH 5.5 to 6.0, but at pH 3.5 and above pH 6.5 the enzyme was very unstable (17). Since rennin is a decomposition product of a protein of the acid albumin type (16) it would seem that instability of rennin at pH 3.5 was due to autolysis of the enzyme. Above pH 6.5 the enzyme lost milk clotting activity rapidly probably due to pH denaturation. Mickelsen and Ernstrom (34) found great stability of crystalline rennin solutions at pH 5.0 to 6.0 and regions of rennin instability around pH 3.8 and above pH 6.5 confirming the work of Foltman.

Experiments by Mickelsen and Ernstrom (34) showed that chloride ions had a negative effect on the stability of crystalline rennin solutions below pH 5.0, but no effect above that pH. Below pH 4.5 the destructive effect of chloride ions on rennin stability was very pronounced.

Struble and Sharp (49) found that rennin in whey was most stable to heat treatments at pH 4.0. At pH values higher and lower than 4.0 rennin activity decreased.
rapidly as the temperature and time of heating increased.
The work of Foltman (17) and Mickelsen and Ernststrom
(34) showed rennin to be fairly stable below pH 3.5
in the absence of chloride ions and very unstable around
pH 3.5-3.8, thus casting strong doubts on the validity
of the report by Struble and Sharp (49).
Studies of rennin digestion by several proteases
have been conducted by Tauber and Kleiner (50). They
found that pepsin can digest 4,425,000 units\(^1\) of rennin
within 18 hr and that trysin can digest the same amount
within 10 hr. Erepsin did not digest any rennin within
24 hr. Foltman (17) and Mickelsen and Ernstrom (34)
have reported evidences of self digestion by rennin.
The role of residual rennin in cheese ripening.
Killing (25) found that residual rennin, capable of clot-
ting milk, remains in cheese. The role of residual rennin
in cheese ripening has been studied extensively.
Sherwood (44) and Van Slyke (52) found increased
soluble nitrogen in cheese made with higher-than-normal
amounts of rennet. Freeman (19) reported more soluble
nitrogen in cheese made with rennet plus pure rennin
that in that made with rennet alone. Sherwood (44)
added chloroform to cheese curd at the stage of salting
to destroy microorganisms and then followed the soluble
and non-protein nitrogen to determine protein breakdown

\[^1\) 1 unit was the mg of milk clotted by 1 mg of
rennin at 40°C in 10 minutes at pH 6.2.\]
during curing. He assumed that all the microorganisms had been killed by the chloroform and concluded that protein breakdown was due almost entirely to the proteolytic action of rennet enzymes. However, Babel and Somkuti (2) and Ernstrom et al. (14) found that the level of rennet used in making Cheddar cheese always influenced the amount of soluble nitrogen developed during curing. Never-the-less changing the rennet concentration by a factor of two did not result in a two-fold change in soluble nitrogen in the cheese during curing. Further more, Ernstrom et al. (14) found more soluble nitrogen developed in cheese made from pasteurized milk than when made from pasteurized milk. The findings of Babel and Somkuti (2) and Ernstrom et al. (14) indicate that the conclusion arrived at by Sherwood (44) was overstated and that although rennet contributed to proteolysis in cheese during curing, most protein breakdown was caused by bacterial proteases.

Linklater and Ernstrom (29) found more soluble nitrogen in cheese made with crystalline rennin than in that made with commercial rennet. They attributed the increased proteolysis of cheese casein to rennin.

Melachouris and Tuckey (32) showed that pepsin in commercial rennet extracts was not the factor responsible for major proteolysis in cheese as suggested by Babcock, Russell and Vivian (1). Cheese made under various conditions of pH exhibited more proteolysis when made with rennet than when a pepsin preparation was used.
Methods for measuring rennin activity. Various methods have been tried to obtain accurate measurements of rennin activities.

The earliest tests for measuring rennin activity were based on the time required for the enzyme to clot milk. Even today the most frequently used tests for measuring rennin activities of many enzymes are based on milk clotting times.

Since milk varied widely in composition, especially in mineral salt content such as calcium, a substrate, uniform in composition, was needed to measure rennin activities (21). Berridge (6) proposed a substrate made by reconstituting 12.0 g of nonfat dry milk powder in 10 ml of .01M CaCl₂. Test tubes, containing 10 ml of the substrate each, were placed in a 30°C water bath for 30 to 35 min and inoculated with 1 ml of diluted rennet extract. The endpoint was determined by noting the time required for a film of milk on the inside surface of the test tube to break into tiny flakes. The technique for determining the endpoint of the enzyme reaction was later improved by Berridge (4).

Sommer and Matsen (46) described a machine which they used to test rennin activity on mastitic milks. The apparatus consisted of a retangular metal box containing water held at constant temperature (30°C). Running across the width of the box were rotating (8 rpm) metal rods making an angle with the horizontal. The angle was such
(20 degrees) that when a 135 ml wide-mouth bottle was placed between two adjacent rods it was partially submerged in the water. To test mastitic milk for clotting time, 50 ml of the milk at 30 C were inoculated with 1 ml of a 1:50 dilution of commercial rennet extract. The wide-mouth bottles containing rennet-treated milk were placed between two adjacent rotating rods of the apparatus and allowed to clot. Time of clotting was measured by timers attached to the apparatus and started when the milk samples were inoculated. The end of the reaction was taken as the time when a thin film of milk adhering to the inside surface of the bottle broke into small flakes of curd.

Bakker, Scheffers and Wiken (3) proposed an apparatus similar to the one by Sommer and Matsen for testing milk clotting activities.

Using the rennet tester of Sommer and Matsen, Ernstrom (10) found that the clotting time of Berridge substrate (6) continued to increase for 20 hr after preparation when it was stored at 2 C. He suggested that the substrate be stored at 2 C for 20 hr before use.

Ernststrom (11) combined the Sommer-Matsen apparatus (46) and the Berridge substrate (6) to measure rennin activities. The activity in a rennet extract of unknown strength was measured by the time required for 1 ml of an appropriate dilution of the unknown extract to clot 25 ml of Berridge substrate. This was compared to the time
required for 1 ml of a known dilution of a standard strength rennet extract to clot 25 ml of an identical substrate run at the same time in the tester. The strength of the undiluted standard was arbitrarily assigned the value of 100 rennin units (RU) per milliliter. The activity in the unknown was related to the standard by the equation:

\[ \text{RU/ml} = 100 \frac{T_s}{T_u} \times \frac{C_s}{C_u} \]

where RU = rennin units

Ts = coagulation time of standard

Tu = coagulation time of unknown

Cu = concentration of unknown

Cs = concentration of standard

100 = rennet units in 1 ml of undiluted standard

Gorini and Lanzavecchia (20) proposed substrates at pH 7.7 and 5.8 for measuring milk clotting activities of bacterial proteases. The pH 7.7 substrate was made by combining 40 ml of \(6.6 \times 10^{-2}\) M cacodylic acid, 60 ml \(6.6 \times 10^{-2}\) M triethanolamine, 1 ml 3M CaCl\(_2\) and 1 g NDM. To prepare the pH 5.8 substrate the volumes of cacodylic acid and triethanolamine solutions used were changed to 70 and 30 ml respectively. The substrates were stored for 24 hr at 37 C before use.

Wang (54) modified the pH 5.8 substrate of Gorini and Lanzavecchia (20) and used it to measure low levels of rennin activities in cheese. The substrate was prepared by combining 50 ml of 0.5M cacodylic acid, 30 ml of
0.2M triethanolamine and 50 ml of 0.2M CaCl₂, making up to 500 ml volume with distilled water and reconstituting 6 g of low-heat NDM in the solution. The final pH of the substrate after 20 hr storage at 20°C was 5.8. The substrate was much more sensitive than the Berridge substrate.

Attempts have been made to determine clotting times of milk substrates automatically. An automatic blood-clot timer was used by De Man and Batra (9) to measure milk clotting activities of rennet solutions. Storrs (48) built an apparatus that could measure the clotting time of a substrate of NDM in distilled water. Everson and Winder (15) used an instrument for measuring sound velocities in liquids to measure rennet coagulation time in milk. They found that there was a large increase in sound velocity when coagulation of a milk substrate occurred.

When milk is treated with rennin a drop in viscosity is first observed due to the action of the enzyme on casein, then suddenly a great increase in viscosity brought about by the clustering of casein micelles (35, 42). The initial phenomena has been used as a basis for measuring the action of rennin on milk (35, 41, 42). A difficulty that often arises when rennin activity is measured by this method is that it is hard to determine a true drop in viscosity. At some very early point during rennin action on casein, coagulation is very
likely occurring on such a small scale that it goes unobserved. This overlapping increases with time until a point is reached when so much paracasein (casein acted on by rennin) is present that coagulation occurs and overshadows any decrease in viscosity. To avoid incipient coagulation of paracasein, Oosthuizen (35) used solutions of sodium caseinate as substrates for rennin action. He found that the rate in drop of viscosity was dependent on the amount of kappa casein present. A difference in various calf rennets was also observed and attributed to a difference in protease content between the extracts.

Lawrence and Sanderson (27) reported a procedure for measuring microquantities of proteolytic enzyme activities. Thin layers of calcium caseinate in agar were prepared by depositing 1 ml of melted agar-calcium caseinate solution on one square inch areas of a microscope slide. A 2 mm diameter well was made in the center of the agar gel and diluted enzyme solutions placed in the well. Proteolytic activities were related to the diameter of a precipitated zone produced by the enzyme. However, the procedure they reported did not work (J. Reyes, personal experiments).

Hill (22) found a pentapeptide and a sulfite ester that can serve as substrates for rennin. The peptide N-ser-leu-phe-meth-ala-o-me (methylester) is always split between the phe-meth bond. Peptides such as phe-meth and others not having the appropriate sequence of amino acids
were not attacked by rennin. Phenyl sulphite ester is also attacked by rennin.

The pentapeptide described by Hill (22) is ideal for determining the loss of activity of crystalline rennin over a period of years but because of the difficulty in preparing the peptide it would be highly impractical as a routine substrate to measure rennin activities.
METHODS OF PROCEDURE

Measuring rennin activity. A substrate proposed by Wang (54) was used to measure rennin activities (milk clotting). The substrate was prepared by mixing 6 g of low-heat NDM with 500 ml of a solution containing 0.02 M CaCl₂, 0.05 M cacodylic acid and 0.012 M triethanolamine. The final pH of the substrate was 5.8. The substrate was stored for 18-36 hr at 2°C before use.

A rennet tester similar to the apparatus introduced by Sommer and Matsen (46) was used for activity measurements. Electrically operated counters operating at 20 counts per minute were used to measure coagulation time in counts. Twenty-five milliliters of cold substrate were placed in a 125 ml wide-mouth bottle and held for 30 min at 30°C in a water bath. Two milliliters of whey or filtrate from a curd slurry preparation were added to the tempered substrate. The time for the whey or filtrate to produce visible coagulation in the body of the substrate was compared to the time required for a known dilution of a standard rennet extract to cause the same effect on the substrate under identical conditions. All tests were run in duplicate and standard rennet extract dilutions were each time
tested simultaneously with the whey or filtrate. Through appropriate dilutions of standard rennet extract all coagulation times produced by the standard were made to occur within ± 10 per cent of the coagulation times induced by the whey or filtrate samples.

A standard rennet extract (SRE) was arbitrarily assigned the value of 100 rennin units (RU) per milliliter and was used as a reference for all measured rennin activities. The strength of the standard was such that 1 ml of a 1:250 dilution produced visible clotting in 25 ml of Berridge (6) substrate (tempered for 20 min at 30 C) in 125 ± 6 counts. To relate a solution containing unknown rennin activity to the SRE, duplicates of both the unknown solution and the diluted SRE were run at the same time on the rennet tester. The coagulation time and dilution of each enzyme solution was noted. Activity in the unknown solution was calculated in terms of RU per milliliter by the equation proposed by Ernstrom (11).

Preparation of whey and curd for testing. Fresh raw milk was pasteurized at 63 C for 30 min and stored for 15 hr at 2 C. The cold milk was quickly warmed to 22 C in a water bath at 50-60 C, and the pH adjusted to the desired value with 1 M HCl. The milk was divided into 454 g samples and warmed to 30 C in a water bath. Each sample was inoculated with 10.6 ml of rennet solution
containing 6.7 RU, and immediately decanted into two 250 ml centrifuge bottles. The samples were allowed to stand at 30 °C for 15 min beyond the first appearance of coagulation. The curd was broken by rapidly shaking the centrifuge bottles twenty-one times. The resultant curd-whey slurry was centrifuged in a number 1 International Centrifuge with conical head at top speed for 20 min to separate the whey and curd. The whey was decanted into a preweighed 500 ml graduated cylinder and the weight and volume noted. The weight of the curd was determined by subtracting the weight of the whey from the total weight of the milk sample plus the weight of enzyme solution added. The volume of the curd was similarly determined by subtracting the volume of the whey from the volume of the milk plus rennet. The whey was stored in the refrigerator at 2 °C until it was ready to test.

The curd was removed from the centrifuge bottles with a spatula and ground with a mortar and pestle. Thirty grams of well-mixed ground curd were placed in a Waring Blender and mixed with the desired amount of double distilled water at low speed for 2 min to produce a curd slurry. Where necessary pH adjustments of the curd slurries were made in the Waring Blender jar by adding 0.5 N NaOH. The volume of alkali used to adjust the pH was included as part of the curd dilution. A portion
of the curd slurry was filtered through Grade 637 coarse filter paper and the filtrate tested for rennin activity.

**Source of milk coagulants.** Rennet extract for this study was obtained from New Zealand Cooperative Rennet Company, Eltham, New Zealand. This rennet was manufactured completely from vells taken from 3 to 6 day old calves. Crystalline rennin, prepared by the method of Ernststrom (11), was obtained from Pfizer Research Laboratories, Milwaukee, Wisconsin.

Standard rennet extract (SRE) was obtained from Dairyland Food Laboratories, Inc., Waukesha, Wisconsin.

**Source of milk and subsequent treatments.** All milk used in this study was obtained from the Utah State University Dairy Farm. The milk was pasteurized at 63 C for 30 min and stored at 2 C for at least 15 hr to allow milk constituents to arrive at equilibrium.
RESULTS

Effect of age of substrate on coagulation time. Freshly prepared substrate was stored at 2 C and tested with identical amounts of rennet at intervals between 1 and 36 hr. Coagulation was induced by inoculating 25 ml of substrate (tempered for 20 min at 30 C) with 2 ml of a 1:5000 dilution of SRE in water.

The coagulation time of the substrate changed very rapidly with aging during the first 10 hr. After 18 hr the rate of change decreased substantially, but coagulation time continued to increase slowly up to 36 hr.

Fresh dilutions of standard rennet extract were prepared each time the substrate was tested to avoid deterioration of the rennet in dilute solution. Each activity measurement was run in duplicate. The results are shown in Figure 1.

Effect of tempering on the coagulation time of the substrate. Substrate was prepared and stored for 18 hr at 2 C. Ten samples of substrate were placed in the rennet tester and the coagulation time measured at intervals between 10 and 120 min. Coagulation was induced by inoculating each sample with 2 ml of SRE diluted 1:5000 in water.

Fresh dilutions of SRE were prepared each time the substrate was tested. Each test was run in duplicate.
Figure 1. Effect of storage at 2 C on coagulation time of substrate
There was a rapid change in coagulation time during the first 30 min of tempering. Between 30 and 60 min there was very little change in coagulation time. The results are given in Figure 2.

Heat inactivation of rennet in whey at pH 5.20.

One pound (454 g) of pasteurized milk at pH 5.20 and 30 C was inoculated with 26.8 RU of rennet extract and allowed to stand for 15 min after the first sign of coagulation. The clot was broken and the whey and curd separated by centrifugation. The whey was divided into four 50-ml samples and heated for 5 min at temperatures of 25, 63, 68, and 73 C respectively. Following the heat treatment, each sample was cooled to 25 C and tested for rennin activity. Results are presented in Table 1.

Table 1. Estimate of the percentage of original rennin activity remaining in whey at pH 5.20 following 5 minute heat treatment at various temperatures

<table>
<thead>
<tr>
<th>Temperature (Degrees C)</th>
<th>Coagulation time (Counts)</th>
<th>Rennin activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>126</td>
<td>100.0</td>
</tr>
<tr>
<td>63</td>
<td>263</td>
<td>47.7</td>
</tr>
<tr>
<td>68</td>
<td>1435</td>
<td>8.8</td>
</tr>
<tr>
<td>73</td>
<td>22348</td>
<td>0.5</td>
</tr>
</tbody>
</table>
Figure 2. Effect of tempering time on coagulation time of substrate
The per cent of original rennin activity was determined by setting the coagulation time (in counts) induced by the unheated (25 C) whey equal to 100 per cent. All other coagulation times were related to it by the equation:

\[ E \times t = K \]

Where \( E \) = enzyme concentration (activity)
\( t \) = coagulation time
\( k \) = constant

The 73 C heat-treated whey failed to clot the substrate in 18.6 hr.

It is recognized that this equation does not hold exactly for the coagulation reaction, but the results were adequate for establishing the heat treatment necessary to destroy rennet in whey at pH 5.20.

In subsequent experiments, heat treatments at 73 C for 10 min were used to destroy rennet in whey and curd slurry supernatant. This allowed an extra 5 min treatment as a safety factor.

**Effect of diluting standard rennet extract with whey on the coagulation time of the substrate.** Accurate measurement of rennin activity in whey requires that substances in the whey other than rennet capable of affecting the coagulation time of the substrate must be added to the rennet standard in like concentration. The possibility that non-rennet substances in whey might affect substrate coagulation time was investigated.
Dilution of rennet standard with whey required that all residual rennin activity in the whey be destroyed.

Whey was obtained by clotting 1 lb of milk (at pH 5.20 and 30 C) with 6.7 RU rennet extract. The whey and curd were separated by centrifugation. The whey was heated to 73 C for 10 min and immediately cooled to 25 C.

Five 100 ml volumetric flasks were filled to approximately 5/6 volume with heat-treated whey and 5 were filled with water. To each of the 10 flasks was added 2 ml of 1:100 diluted standard rennet in water. The samples were made to volume with whey and water respectively. The diluted samples (1:5000) were tested simultaneously on the rennet tester. The coagulation time of each sample was measured and reported in Table 2.

A definite difference between the coagulation times of the substrate was observed when coagulation was induced with standard rennet diluted with whey as opposed to standard rennet diluted with water. This emphasized the necessity of diluting all standard rennet samples with heated whey when used as a basis for measuring residual rennin activity in whey samples.

Effect of diluting standard rennet extract with curd slurry supernatants on the coagulation time of the substrate. Curd was obtained from milk clotted at pH 5.20 with 6.7 RU of rennet extract per pound of milk.
Table 2. Effect of diluting standard rennet extract with whey (pH 5.20) and water on coagulation time of substrate

<table>
<thead>
<tr>
<th>Coagulation time</th>
<th>Diluted with whey (counts)</th>
<th>Diluted with water (counts)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>108</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>107</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>109</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>110</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>110</td>
<td>90</td>
</tr>
</tbody>
</table>

Thirty grams of curd were blended with 150 ml of water for 2 min at low speed in a Waring Blender. The curd slurry was centrifuged for 20 min to remove curd particles. The supernatant was heated for 10 min at 73 C and immediately cooled to 25 C. Clotting activities of standard rennet diluted with curd slurry supernatants and with water were compared on the substrate under identical conditions.

The experiment was also carried out by diluting standard rennet with curd slurry obtained from milks clotted at pH 6.00 and 6.65.

No difference in substrate coagulating ability was observed between standard rennet diluted with curd slurry supernatants or with water.
These results indicate that standard rennet used for testing whey must be diluted with whey, and that standard rennet used for testing curd supernatants can be diluted with water.

An increase in pH was always observed when curd slurries were prepared in water. This was probably due to solubilization of colloidal calcium phosphate (Table 3).

Table 3. Effect of diluting standard rennet extract with curd slurry supernatants* and water on the coagulation time of substrate

<table>
<thead>
<tr>
<th>Supernatant pH 5.85 Water</th>
<th>Supernatant pH 6.55 Water</th>
<th>Supernatant pH 7.10 Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Counts</td>
<td>Counts</td>
<td>Counts</td>
</tr>
<tr>
<td>89</td>
<td>89</td>
<td>89</td>
</tr>
<tr>
<td>91</td>
<td>89</td>
<td>89</td>
</tr>
<tr>
<td>91</td>
<td>90</td>
<td>89</td>
</tr>
<tr>
<td>92</td>
<td>90</td>
<td>89</td>
</tr>
<tr>
<td>91</td>
<td>91</td>
<td>91</td>
</tr>
</tbody>
</table>

* Slurry supernatants prepared from curd formed at pH 5.20, 6.00, and 6.65.

Effect of added curd on the coagulation time of the substrate. Curd was obtained from milk clotted at pH 5.20 with 6.7 RU of rennet extract per pound of milk. After separation from whey, 30 g of curd were blended
with 450 ml of water. The slurry was adjusted to pH 6.80 with 0.5N NaOH, and curd particles in the slurry were removed by filtering on coarse filter paper².

Each of two 25 ml samples of substrate at 30°C was inoculated with 2 ml curd slurry filtrate. Immediately following inoculation, about .25 g curd (obtained from the filtered slurry) was added to one sample of substrate. The curd was allowed to remain in the substrate for 10 min before it was removed by filtration. The sample without added curd was also filtered to compensate for any cooling that may have occurred during filtering.

Addition of curd increased the coagulation time of the substrate as shown in Table 4.

Table 4. Effect of added curd on the coagulation time of substrate

<table>
<thead>
<tr>
<th>Coagulation time</th>
</tr>
</thead>
<tbody>
<tr>
<td>No curd added</td>
</tr>
<tr>
<td>(counts)</td>
</tr>
<tr>
<td>305</td>
</tr>
<tr>
<td>313</td>
</tr>
<tr>
<td>321</td>
</tr>
</tbody>
</table>

2. Coarse filter paper removed curd particles from slurry much faster than centrifugation.
The longer coagulation time may have been due to removal of calcium ions from solution by the added curd which would have made the substrate less sensitive.

**Effect of pH at setting on total rennin activity**

accounted for in curd and whey. Three lots of milk were adjusted to pH 5.20, 6.00 and 6.65 (normal pH of milk) respectively with 1M HCl. Water was added to some samples to compensate for the maximum volume of HCl used. Consequently the same amount of liquid was added to all samples. A 454 g sample was taken from each lot and clotted with 6.7 RU of rennet extract at 30 C. The samples were allowed to stand for 15 min after the first appearance of coagulation. The clot was broken and the whey and curd separated by centrifugation.

Activity in the whey was tested by comparing its clotting activity with that of a known whey dilution of rennet extract.

A curd slurry was prepared by blending 30 g of curd in 150 ml of water. A portion of the slurry was filtered and the filtrate immediately tested for rennin activity as previously described. Results are given in Figure 3.

When milk was coagulated at pH 5.20 only 36% of the rennet added to the sample was accounted for in the curd and whey; 15% in the curd and 21% in the whey. When coagulated at pH 6.00, 61% of the activity was accounted for; 22% in the curd and 39% in the whey.
Figure 3. Effect of pH at setting on total rennin activity recovered from whey and curd using a 1:5 dilution of curd in water.
When milk was coagulated at pH 6.65, 83% of the activity was accounted for; 22% in the curd and 61% in the whey.

**Effect of pH adjustments of curd slurries on recovery of rennin activity from curd.** Curd was obtained from milk coagulated at pH 5.20 and 30 C with 6.7 RU of rennet extract per pound of milk.

Thirty grams of curd were blended in a Waring Blender with 150 ml of water to make a curd slurry. Three 50 g samples were taken from the curd slurry and adjusted to pH 5.75, 6.00 and 6.80 respectively with 0.5N NaOH. The samples were filtered to remove curd particles from the slurries and the filtrates tested for activity. See Figure 4.

The theoretical activity in the curd was calculated by subtracting the activity measured in the whey from the total activity added to clot the milk sample. Increasing the pH values of curd slurries resulted in higher rennet activities measured in the curd slurry filtrates. Rennin appeared to be bound to the curd at low pH values and was released when the pH was increased from 5.75 to 6.80.

**Effect of dilution with water on the total rennin activity recovered from curd.** Curd was obtained from milk clotted at pH 5.20 and 30 C with 6.7 RU of rennet extract per pound of milk, and separated from the whey by centrifugation.

Thirty grams of curd were blended in a Waring Blender
with the desired volume of water. The pH of the slurry was adjusted to 6.80 with 0.5N NaOH and filtered.

Maximum activity was recovered when the curd was diluted 16 times with water. Dilutions smaller and greater than 16 resulted in smaller activity recoveries (Figure 5). Increasing dilutions may have exposed more curd surface to the water releasing rennin into solution which accounted for the greater activity measured as dilution was increased. Beyond the 16 dilution the rennin appeared to be unstable during blending. Rennet extract diluted with water lost approximately 60% of its activity during 2 min blending at low speed (J. Reyes, unpublished data).

Effect of incubation of curd slurry on the recovery of rennin activity from curd. Curd was obtained from milk clotted at pH 5.20 and 30°C with 6.7 RU of rennet extract per pound of milk and separated from the whey by centrifugation.

Thirty grams of curd were blended in a Waring Blender with 450 ml of water. The pH of the slurry was adjusted to 6.80 and incubated at 25°C. Small portions of the slurry were filtered at intervals between 10 and 120 min, and the filtrates tested for rennin activity. Results are presented in Table 5.

Maximum activity from the curd appeared to occur after 30 min incubation, but differences in activities between 10 and 120 min were not significant.
Figure 5. Effect of curd dilution at pH 6.8 on amount of rennin activity recovered from the curd.
Table 5. Effect of incubation (25 C) of curd slurry (pH 6.8) on recovery of rennin activity from curd

<table>
<thead>
<tr>
<th>Incubation time (min)</th>
<th>Rennin activity in curd (RU/100 g curd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>6.5</td>
</tr>
<tr>
<td>30</td>
<td>6.6</td>
</tr>
<tr>
<td>60</td>
<td>6.4</td>
</tr>
<tr>
<td>120</td>
<td>6.4</td>
</tr>
</tbody>
</table>

Total rennin activities recovered from whey and curd prepared from freshly coagulated milk. Milk at 22 C was adjusted to pH 5.20 with 1M HCl and divided into 10 one-pound samples. Each sample was warmed to 30 C and inoculated with 6.7 RU of rennet extract. After the first appearances of coagulation each sample was allowed to stand for 15 min. The clot was broken by agitation and the whey and curd separated by centrifugation.

Activity in the whey was measured by comparing its activity to that of a known standard rennet extract diluted with heat-treated whey. A curd slurry was prepared by blending 30 of curd in 450 ml of water. The pH of the slurry was adjusted to 6.80 with 0.5N NaOH and allowed to incubate for 30 min at 25 C. Curd particles were removed from the slurry by filtration and
the filtrate tested immediately. Activity in the curd filtrate was measured by comparing its activity to that of a known rennet standard diluted with water.

The activity measured in the whey was subtracted from the total activity added to the milk and the difference was considered the theoretical activity in the curd. The theoretical activities in 10 samples of curd were measured by using a 1:15 dilution of curd in water and adjusting the curd slurries to pH 6.80. Results are presented in Table 6.

An average of 89 ± 2.1% of the theoretical activity in the curd was accounted for by this procedure and the total activity recovered from curd and whey was 91 ± 1.6% of that added to the milk.

The experiment was also repeated with crystalline rennin since the presence of small amounts of pepsin in rennet extract could have affected the results. Pepsin is not very stable above pH 6.5. Four 454 g samples of pasteurized milk (pH 5.20 and 30 C) were clotted with 6.7 RU of crystalline rennin each, and the whey and curd from the samples tested for rennin activity. Results are presented on Table 7. No significant difference in total rennin activity recovery was noted when crystalline rennin or New Zealand rennet extract was used to clot the milk samples. A slight difference in distribution of enzyme between the
Table 6. Recovery of rennin activity from curd and whey prepared from ten samples of milk (454.0 g) clotted at pH 5.20 with 6.7 RU of rennet extract*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Whey (g)</th>
<th>Curd (g)</th>
<th>Activity measured in whey (RU)</th>
<th>Theoretical activity in curd (RU)</th>
<th>Activity measured in curd (RU)</th>
<th>Per cent of theoretical (%)</th>
<th>Total recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>385.6</td>
<td>79.0</td>
<td>1.4</td>
<td>5.3</td>
<td>4.5</td>
<td>85</td>
<td>88</td>
</tr>
<tr>
<td>2</td>
<td>385.6</td>
<td>79.0</td>
<td>1.5</td>
<td>5.2</td>
<td>4.7</td>
<td>90</td>
<td>92</td>
</tr>
<tr>
<td>3</td>
<td>397.9</td>
<td>66.7</td>
<td>1.5</td>
<td>5.2</td>
<td>4.7</td>
<td>90</td>
<td>92</td>
</tr>
<tr>
<td>4</td>
<td>396.6</td>
<td>68.0</td>
<td>1.5</td>
<td>5.2</td>
<td>4.7</td>
<td>90</td>
<td>92</td>
</tr>
<tr>
<td>5</td>
<td>391.9</td>
<td>72.7</td>
<td>1.4</td>
<td>5.3</td>
<td>4.7</td>
<td>89</td>
<td>91</td>
</tr>
<tr>
<td>6</td>
<td>393.6</td>
<td>71.0</td>
<td>1.4</td>
<td>5.3</td>
<td>4.6</td>
<td>87</td>
<td>90</td>
</tr>
<tr>
<td>7</td>
<td>388.3</td>
<td>76.3</td>
<td>1.4</td>
<td>5.3</td>
<td>4.5</td>
<td>85</td>
<td>88</td>
</tr>
<tr>
<td>8</td>
<td>387.8</td>
<td>76.8</td>
<td>1.4</td>
<td>5.3</td>
<td>4.8</td>
<td>91</td>
<td>92</td>
</tr>
<tr>
<td>9</td>
<td>390.8</td>
<td>73.8</td>
<td>1.4</td>
<td>5.3</td>
<td>4.8</td>
<td>91</td>
<td>92</td>
</tr>
<tr>
<td>10</td>
<td>390.3</td>
<td>74.3</td>
<td>1.4</td>
<td>4.3</td>
<td>4.7</td>
<td>89</td>
<td>91</td>
</tr>
</tbody>
</table>

Mean Activity measured in curd (RU) 4.7
SD 0.11

Mean Activity measured in curd (RU) 89
SD 2.1

Mean Total recovery (%) 91
SD 1.6

*Rennet extract supplied by New Zealand Rennet Co., Eltham, N.Z.
Table 7. Recovery of rennin activity from curd and whey prepared from four samples of milk (454.0 g) clotted at pH 5.20 with 6.7 RU of crystalline rennin*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Whey (g)</th>
<th>Curd (g)</th>
<th>Activity measured in whey (RU)</th>
<th>Theoretical activity in curd (RU)</th>
<th>Activity measured in curd Total (RU)</th>
<th>Per cent of theoretical (%)</th>
<th>Total recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>394.3</td>
<td>71.3</td>
<td>1.0</td>
<td>5.7</td>
<td>5.2</td>
<td>91</td>
<td>93</td>
</tr>
<tr>
<td>2</td>
<td>392.6</td>
<td>73.0</td>
<td>1.0</td>
<td>5.7</td>
<td>5.0</td>
<td>88</td>
<td>90</td>
</tr>
<tr>
<td>3</td>
<td>392.1</td>
<td>73.5</td>
<td>1.1</td>
<td>5.6</td>
<td>5.0</td>
<td>89</td>
<td>91</td>
</tr>
<tr>
<td>4</td>
<td>392.2</td>
<td>73.4</td>
<td>1.1</td>
<td>5.6</td>
<td>4.9</td>
<td>88</td>
<td>90</td>
</tr>
</tbody>
</table>

Mean Activity measured in whey 1.0 5.6 5.0 89 Total recovery 91
Mean SD 0.0067 0.0067 0.017 1.4

* Crystalline rennin, prepared by the Ernstrom (11) procedure, was obtained from Pfizer Research Laboratories, Milwaukee, Wisconsin.
whey and curd was observed when the different enzyme preparations were used. However, this difference in distribution was not large enough to be significant.

These results seem to indicate that nearly all, if not 100%, of the enzyme in New Zealand rennet extract was actually rennin.
DISCUSSION

When rennin is used as a coagulant for cheese making, the enzyme is partitioned between the curd and whey. Some of it, of course, may be inactivated during the cooking operation, but that which remains active in the curd plays a measurable role in the body and texture development of the cheese during curing.

A test procedure was developed for quantitatively measuring the residual rennin remaining in curd and whey separated from freshly coagulated milk. The milk was coagulated with amounts of rennin that would normally be used in making Cheddar cheese, and total recovery in the curd and whey together amounted to 91 ± 1.6% of the activity added. The test was a coagulation test in which a very sensitive substrate proposed by Gorini and Lanzavecchia (20) and modified by Wang (54) was used.

The endpoint of the enzyme reaction was indicated by the sudden appearance of tiny flakes in the body of the substrate. Its sensitivity was such that 2 ml of a 1:5000 dilution of SRE in water coagulated 25 ml of substrate (30 °C) in 89 ± 1 count (20 counts/min) of the rennet tester. Compared to the Berridge (6) substrate, the new substrate was approximately 12-15 times more sensitive.
The test procedure was developed on fresh coagulum rather than on cheese curd because recovery of activity required conditions that would not result in enzyme inactivation which might occur during cheese making.

The distribution of rennin between curd and whey and the release of rennin by curd was profoundly affected by pH. As the pH of the milk at coagulation decreased, more rennin was bound to the curd and less released into the whey. Subsequent adjustment of the pH of curd-water slurries to pH 6.80 resulted in release of substantially higher levels of rennin from the curd. This suggests that there is probably more rennin left in cheese curd when the pH at coagulation is low and less when it is relatively high.

The substrate used in this test was extremely sensitive, and accurate results required that non-rennin substances in the whey which affected the coagulation time had to be added in equal quantity to the reference standard. Soluble matter in curd slurry supernatants did not affect the substrate. However, curd particles retarded coagulation and had to be filtered out. Whey heated to 75 C for 10 min and cooled to 25 C was used to make a dilution of SRE which served as a standard of comparison for measuring rennin activity in whey from freshly coagulated milk. Heat treatment of the whey caused precipitation of whey proteins and calcium phosphate.
The effect on the coagulation time of the substrate by using heat-treated whey to make SRE dilutions as opposed to making the dilutions with non-heated rennin-free whey was not studied. Since soluble phosphate in whey definitely affects the sensitivity of calcium rich substrates and since the heat treatment of whey precipitates the soluble calcium phosphate, there remains the possibility that non-rennin substances in SRE diluted with heat-treated whey still did not affect the substrate exactly as the non-rennin substances in unheated whey. This effect should be investigated in future studies.

Mixing curd slurries at pH 6.80 with increasing amounts of water in a Waring Blender at low speed for 2 min had a pronounced effect on the release of rennin activity from curd. Dilutions of curd greater than 1:15 resulted in loss of activity.

An earlier experiment revealed that when a 1:4540 dilution of SRE in pure water was blended at low speed for 2 min in a Waring Blender, as much as 60% of the original rennin activity was lost. The experiment also showed that when a similar dilution of SRE was made in curd slurry supernatants (1:15 dilution) and blended at low speed for 2 min there was no loss in rennin activity. Results of the experiment indicated that in curd dilutions greater than 1:15 the curd slurries became more and more like water, and blending apparently
had a destructive effect on the rennin that was released from the curd.

**Recommended procedure for preparing substrate.**

1. Prepare stock solutions of 0.5M cacodylic acid, 0.2M CaCl₂, and 0.2M triethanolamine.

2. Prepare a buffer solution by placing 50 ml 0.5M cacodylic acid, 50 ml 0.2M CaCl₂, and 30 ml 0.2M triethanolamine into a 500 ml volumetric flask and make to volume with distilled water. Mix 6 g of low-heat NDM into 500 ml of the prepared buffer solution.

3. Store the substrate at 2°C for 18-36 hr before use.

**Recommended procedure for measuring rennin activity in whey.**

1. Heat a portion of the whey to be tested to 75°C for 10 min and immediately cool to 25°C with cold water. Use this whey to make an appropriate dilution of a standard rennet extract.

2. Pipette 25 ml of aged substrate into each of two 125 ml wide-mouth test bottles, and temper in a 30°C water bath for 30 min.

3. Inoculate the substrate in the separate test bottles with 2 ml of whey and 2 ml of diluted standard rennet respectively. Duplicates of each are recommended.

4. Start timing immediately upon inoculation of the substrate. The endpoint is taken as the time when tiny curd flakes first appear in the body of the substrate. The coagulation time of the known rennet dilution should be within ±10% of the coagulation time of the unknown. When necessary this can be adjusted by changing the dilution of the standard rennet.

5. The rennin activity in the unknown is related to the standard rennet by the equation proposed by Ernstrom (13):
\[
\text{RU/ml} = 100 \frac{\text{Ts}}{\text{Tu}} \times \frac{\text{Du}}{\text{Ds}}
\]

where
- \(\text{RU}\) = rennin units
- \(\text{Ts}\) = coagulation time of the standard (known)
- \(\text{Tu}\) = coagulation time of the unknown
- \(\text{Ds}\) = dilution of the standard (known)
- \(\text{Du}\) = dilution of the unknown
- 100 = rennin units per ml of undiluted standard (known)

**Recommended procedure for measuring rennin activity in curd.**

1. Determine the volume of a 30 g sample of fresh curd (subtract the volume of whey from the volume of original milk).

2. Place the sample in a Waring Blender with 450 ml distilled water and blend at low speed for 2 min to obtain a curd slurry.

3. Adjust the curd slurry to pH 6.80 with 0.5N NaOH while the slurry stirs at low speed in the Waring Blender jar. Note the volume of NaOH and add this to the dilution of the curd.

4. Allow the curd slurry to stand at 25°C for 30 min. Filter a small portion of the curd slurry to obtain curd-free filtrate. Prepare an appropriate dilution of standard rennet in distilled water.

5. Pipette 25 ml of aged substrate into each of two 125 ml wide-mouth test bottles, and temper in a 30°C water bath for 30 min.

6. Inoculate the substrate in the separate test bottles with 2 ml of curd slurry supernatant and 2 ml of diluted standard rennet respectively. Duplicates of each are recommended.

7. Start timing immediately upon inoculation of the substrate. The endpoint is taken as the time when tiny curd flakes first appear in the body of the substrate. The coagulation time of the known rennet dilution should be within ±10% of the coagulation time of the unknown. When necessary this can be adjusted by changing the dilution of the standard rennet.
8. Calculate rennin activity as in step 5 of the whey procedure. This will give the activity per milliliter of original curd. This can be changed to activity per gram since both the weight and volume of the original curd are known.
BIBLIOGRAPHY


