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Procedure for Measuring Residual Endothia Parasitica Protease in Curd and Whey From Freshly Coagulated Milk

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PROCEDURE FOR MEASURING RESIDUAL ENDOTHIA PARASITICA PROTEASE IN WHEY AND CURD FROM FRESHLY COAGULATED MILK

by

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Raman B. Patel

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ABSTRACT

Procedure for Measuring Residual Endothia parasitica Protease in Curd and Whey from Freshly Coagulated Milk

by

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Utah State University, 1973

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Test procedures were developed for measuring the residual milk clotting activity of a protease produced by Endothia parasitica in curd and whey separated from freshly coagulated milk. A substrate was prepared by reconstituting 6 g low heat nonfat dry milk in 500 ml buffer containing 50 ml 0.5M cacodylic acid, 50 ml 0.2M $CaCl_2$, 30 ml 0.2M triethanolamine and 370 ml double distilled water. The substrate was stored at 2 to 4 C for 20 hours before use. Two milliliters of whey or supernatant from centrifuged curd-water slurries were inoculated into 25 ml of substrate at 30 C and the coagulation time noted, and compared with that produced by a known dilution of a standard enzyme solution.

Endothia parasitica curd formed at pH 6.7 contained 45 per cent of the enzyme activity added to 454 g milk but when formed at pH 5.2 the curd contained only 25 per cent.

The recovery of Endothia parasitica protease in curd was made by preparing a 1:5 curd-water slurry, adjusting to pH 5.4, filtering and testing the filterate.

(69 pages)

INTRODUCTION

Rennin has been used as a milk coagulant in cheese making for many years. The principal function of rennin is to catalyze the formation of curd, but it also affects cheese ripening (3, 20). Ernstrom, Price and Swanson (17), Sherwood (78) and Van Slyke and Hart (86) have shown that the amount of rennet extract added to cheese milk affects the rate of proteolysis of the cheese during curing.

A number of milk coagulating enzymes from microbial (3, 26, 65, 71, 89) plant (43, 58) and animal sources (54) have been proposed as substitutes for rennin, but many of these have a marked effect on the flavor and body of the cheese. Most of them produce bitter flavors due to excessive or abnormal proteolysis.

In view of increasing interest in the effect of various coagulating enzymes on cheese curing, a test capable of measuring residual levels of these enzymes in cheese and whey is greatly needed. Most of the tests for measuring rennin activity are based on the length of time required for a given amount of milk to clot after the addition of a measured amount of diluted enzyme. However, cheese curd contains such a low level of coagulating enzyme that it is not to coagulate normal milk substrate in a reasonable time. A substrate developed by Wang (90) during preliminary studies of residual rennin in Cheddar cheese was sensitive enough to measure the rennin in curd and whey.

The principle objective of this investigation was to develop a technique for quantitatively measuring the amount of a milk clotting enzyme produced by Endothia parasitica in curd separated from freshly coagulated milk. Further aim of this research was to study the effect of pH and other factors on the distribution of this coagulating enzyme between curd and whey.

REVIEW OF LITERATURE

Rennin has been used as a milk coagulant in cheese making for many years (6, 88). It is one of the proteolytic enzymes in the abomasum of milk-fed calves (6, 67, 88). Like other proteolytic enzymes in the digestive tract rennin is secreted as an inactive precursor called prorennin (20, 67). Lorcher (50) found that when acid was added to extracts of the gastric mucosa of calves, there was rapid increase in the milk clotting activity of the extract. This increase in activity was attributed to conversion of inactive prorennin to active rennin (15). Rand and Ernstrom (67) showed that prorennin was activated at pH values between 4.7 and 5.0 mainly by an autocatalytic reaction. Activation of proreenin can also be catalyzed by pepsin at pH values between 5.5 and 6.0 (48).

The cheese industry uses rennin in the form of a crude extract (6, 64), paste (63) or powder (64) called rennet (20).

Manufacture of Commercial Rennet

Calve's stomachs (vells) are generally prepared in the abattoir in one of the two ways: dry-blown or flat salted (20, 88). In the dry-blown process, used mostly in Europe and New Zealand, the contents of the stomach are expressed, and the intact vell tied, inflated with air and dried. In the flat salted process used in the United States, the vells are opened at the slaughterhouse, washed and packed in salt for shipment (20). The salted vells are generally

washed and dried prior to extraction (64). The dried vells in either process are shredded, mixed with an inert filler such as excelsior, saw dust, or glass wool and extracted with a sodium chloride solution (64). The crude extract contains active rennin as well as inactive prorennin (20). Sufficient hydrochloric acid is then added to the crude extract to adjust the pH to 4.7-5.0 to hasten the conversion of prorennin to rennin (64, 88). After clarification and filtration the rennet extract is standardized with respect to activity, salt concentration and pH (64). Commercial rennet should not lose more than 1-2 per cent strength per month when held at pH 5.7 and at a low temperature (13, 32).

Bobeck and Joost (9) reported that most commercial rennet extracts contained 17.25 to 23.8 per cent NaCl and had a pH between 5.4 and 5.9. Placek et al. (64) stated that rennet extract might contain about 5 per cent propylene glycol as a protein stabilizer and 2 per cent sodium propionate and 0.1 per cent sodium benzoate as preservatives.

The proteolytic activity in rennet is attributed to rennin (6, 13, 23, 47) and small amounts of pepsin (6, 13, 47, 48). Leitch (47) pointed out that the enzymatic secretion of very young calves' stomachs was predominantly rennin, but that at the age of 5 months contained pepsin almost to the exclusion of rennin. In 1961 Linklater (48) reported that pepsin contributed up to 3.7 per cent of the clotting strength in commercial rennet extract. It might be more than that at present because rennet is in short supply and more is being extracted from the stomachs of older animals than in 1961. Also to supplement

the supply of rennin, pepsin is often used as a partial substitute in cheese making.

Rennin Stability

Rennin stability is affected by heat (13, 22, 59, 85, 86) ultrasonic vibrations (91), shaking (75), alkali (12, 50, 57, 59, 67, 85) visible and ultraviolet light (42) and by antiseptics like chloroform and formaldehyde (13).

Foltman (23) stated that rennin had maximum stability between pH 5.5 and 6.0. The stability was also good at pH 2.0. However, near pH 3.5 and above pH 6.5 the enzyme was unstable. Mickelsen and Ernstrom (57) reported that above pH 6.0 rennin activity losses increased with increasing pH and were highly temperature dependent. Maximum stability of rennin was maintained between pH 5.0 and 6.0 from 2 C to 30 C in the presence of 0.0 to 0.97 M NaCl. At pH 3.8 maximum instability resulted in a loss of 35 per cent of initial activity at 30 C over 96 hours, but when the ionic strength was increased from 0.03 to 1.0M with NaCl, and other conditions held constant, activity losses increased from 35 to 70 per cent. Under the same conditions of pH, ionic strength and temperature, rennin was more stable in sodium citrate, sodium lactate and ammonium sulfate than in sodium chloride and potassium chloride. However, above pH 5.0 NaCl had very little effect on rennin stability. Activation losses in commercial rennet that occur below pH 5.2 were attributed to autolysis of rennin in the presence of a high concentration of chloride ions.

Struble and Sharp (85) reported that as temperature increased and time of heating was lengthened, the zone of maximum stability (minimum coagulation time) was narrowed. Rennin heated to 70 C for 2 minutes at pH 4.3 in whey showed practically no inactivation, but it was completely inactivated at pH 5.3 or above at the same temperature and time.

Pepsin is present in commercial rennet extract to a limited extent (49). Pepsin activity is more sensitive than rennin activity to pH changes between 6.5 and 6.8 (19). Graber (29) pointed out that the great difference between rennet and pepsin was that "rennet will coagulate sweet milk while pepsin under the same conditions fails more often than it succeeds in producing coagulation." Lucas (51) and Merker (56) suggested that milk should have a titratable acidity of at least 0.2 per cent to work with pepsin for cheese manufacture.

Early attempts were made to replace rennin by pepsin in cheese manufacture (12, 29, 51, 52, 56, 78), but fat losses in the whey were reported greater with pepsin than with rennet (51). Lucas (51), Maragoudakis et al. (52), and Malachouris and Tuckey (54) suggested that pepsin cheese had inferior texture. Reports of bitterness in cheese made with pepsin (13) have not been substantiated in most instances (12, 51, 52, 54, 58). Protein degradation in pepsin cheese was considerably less than in rennet cheese (48, 52, 54, 78). Stine (83) stated that large quantities of "American varieties" of cheese made by using a 75:25 mixture of rennin and pepsin were quite comparable to cheese made with 100 per cent rennet.

A number of milk clotting enzymes from plant and microbial sources have been proposed as replacements for rennin (3, 26, 38, 65, 69, 71, 72, 79, 81, 89, 92). However, most have proved unsuccessful since they produced bitter flavors due to excessive and/or abnormal proteolysis (26, 36, 38, 58, 81). The findings of Nosar (58) indicated that a temperature higher than 50 C was needed for optimum activity of a rennin-like enzyme from higher plants (vegetable rennet). The activity of vegetable rennet fell off rapidly when the temperature changed from 50 C to 37 C, while animal rennet was very active at 37 C. Hence, cheese-making required a larger quantity of vegetable rennet than animal rennet for comparable setting of the milk. The use of higher-than-normal temperatures in cheese making resulted in lower yields of cheese. Cheddar cheese made with an extract from Withania coagulans had good texture but a perceptible bitter taste. Krisnaswami et al. (43) made Cheddar cheese with a milk clotting enzyme from Ficus carica (vegetable rennet). The cheese composition was comparable to that of rennet cheese, but the yield and organoleptic scores were comparatively lower. Cheese made with the vegetable rennet developed a bitter flavor in the early stages of ripening. Windland and Kosikowski (92) also reported that cheese made with vegetable rennet developed a bitter flavor and was not acceptable for consumption.

Richardson et al. (69) stated that a rennin substitute produced from Mucor pusillus var. Lindt was satisfactory for manufacture of cheese such as Brick, Cheddar, Pizza and Parmesan. However, increased fat losses were associated with M. pusillus rennet, and at 14 months Cheddar cheese made

with this fungal rennet developed a bitter flavor. They also found higher lipase activity in the fungal rennet than animal rennet, and suggested this as an advantage in curing. Babel et al. (3) prepared Cheddar cheese with M. pusillus rennet which was criticized for having a rancid flavor and a coarse mealy texture, but protein degradation was less than in rennet cheese. The difference in these two cheese is probably due to var. Lindt which Richardson et al. used and Babel et al. did not. Kikuchi et al. (38) also found a bitter flavor in Gouda cheese made with M. pusillus rennet.

A rennin-like enzyme from Aspergillus usameii was reported to be 3-4 times greater in proteolytic activity than animal rennet. Irvine et al. (36) demonstrated that a rennin-like enzyme from Bacillus subtillis required a higher temperature and longer time for coagulation of milk than equal amounts of animal rennet. Cheddar cheese made with B. subtillis rennet was of excellent quality, but sweet type cheese showed a high degree of proteolysis and off flavor development which was not observed in animal rennet cheese.

Sardinas (71) suggested a mildly proteolytic enzyme elaborated by Endothia parasitica as a rennin substitute. The enzyme had maximum stability near pH 4.5, and an isoelectric point at pH 5.5. Larson and Whitaker (44, 45) reported that over the pH range 5.1 to 6.5, the clotting activity of the E. parasitica enzyme was much less sensitive to the pH change than the activity of rennin. They reported that the enzyme had maximum stability between pH 3.8 to 4.5. At 24.1 C the enzyme was stable from pH 3.0 to 6.5 for 3 hours, but was rapidly inactivated below pH 2.5 and above 7.0.

Shovers and Bavisotto (70) showed that the enzyme from E. parasitica was suitable as a substitute for animal rennet in cheese making. Cheese made with this enzyme was completely acceptable in body and flavor. The enzyme was evaluated in plant trials with Cheddar, Swiss, Monterey, Colby, Limberger and Italian varieties, and could completely or partially replace animal rennet without change in manufacturing procedure. Accelerated flavor and body development were characteristics of cheese made with this enzyme. It scored equally well after 7 months curing with animal rennet cheese cured for 12 months. However, it has been shown that the E. parasitica enzyme produced bitterness in Cheddar cheese. Incubation of fresh ground Cheddar cheese curd with added E. parasitica enzyme produced bitterness after 5 days which was proportional to the amount of enzyme added to the curd (Ernstrom and Wang, unpublished data).

Role of Residual Rennin in Cheese Ripening

Van Slyke (86) found more soluble nitrogen in cheese made with a higher-than-normal amount of rennet. Babcock et al. (1) concluded that increase in the amount of rennet extract used in cheese making increased the soluble nitrogenous substance in the cheese which they suggested as a measure of ripening. They attributed the increase in soluble nitrogen to the pepsin in the rennet extract.

Sherwood (78) found that cheese made with pepsin had considerably less protein degradation than the rennet control cheese. The use of increased

amounts of rennet led to greater protein breakdown during ripening. Sherwood (77) also studied the role of rennet in Cheddar cheese ripening by eliminating bacterial influence with a chloroform treatment. He found that protein degradation was identical in control and chloroform-treated cheese and concluded that rennet was the only important agent in protein degradation during cheese ripening. Babel (2) found a direct relation between the quantity of rennet extract used and the presence of water soluble nitrogen in cheese regardless of the use of $CaCl_2$. Linklater and Ernstrom (49) indicated that crystalline rennin had greater proteolytic activity in cheese than commercial rennet. Therefore, they concluded that rennin was the only essential component of commercial rennet when used for cheese manufacture. It was also reported by Freeman (25) and Peltola and Antila (59) that larger-than-normal amounts of rennet caused cheese to ripen faster and develop a richer flavor than control cheese.

Ernstrom et al. (17) reported that adding 0.02 per cent $CaCl_2$ with one-half the normal amount of rennet extract gave normal curd formation and yield of Cheddar cheese, but the protein breakdown was slow and curdiness persisted longer in the finished cheese. This indicated that rennet played an important role in cheese ripening.

Melachouris and Tuckey (54) demonstrated that Cheddar cheese made with pepsin underwent slower body breakdown and developed less soluble nitrogen during curing than comparable cheese made with rennet. This has also been amply demonstrated in numerous commercial factories. Thus the

conclusion of Babcock et al. (1) that pepsin in rennet extract is responsible for significant proteolysis in cheese is probably not valid.

Role of Micro-organisms in Cheese Ripening

It has been reported that the rate, nature and extent of protein degradation during cheese ripening is affected by bacterial proteases elaborated by starter organisms (11, 33, 35, 37, 41, 61, 76). However, Kelly (37) and Sherwood and Whitehead (76) indicated that streptococci used as starter had little direct influence on the rate of proteolysis in cheese.

The rate of cheese ripening was found to be indirectly influenced by the lactic acid producing bacteria in the milk at the time of setting (30, 31, 34). Van Slyke and Hart (86) observed that the presence of acid in cheese enhanced the action of cheese-ripening enzymes. Sherwood and Whitehead (76) stated that the main trend of protein breakdown was governed by the state and extent of acid formation in the curd during the manufacturing process. They concluded that rennet enzymes were the main proteolytic enzymes involved in cheese ripening and that the main function of streptococci was the formation of acid. The acid influenced the rate of proteolysis, since the maximum proteolytic activity of rennin was near pH 4.0 (23).

Czulak (11), Phillips (62), Price (66), and Staudhouders (82) explained that bitter flavors in cheese were based on the higher proteolytic activity of rennin when excessive acid was produced during manufacture. Staudhouders (82) indicated that the pH affected the distribution of rennin between curd and

whey and in turn affected the flavors of cheese by pH dependent rennin proteolysis which was an indirect effect of micro-organisms.

Presence of Residual Rennin in Cheese

The rennin used to coagulate the milk in cheese manufacture remains functional during ripening. The presence of residual rennin was demonstrated by Barthel et al. (4). They obtained a water extract from cheese by mixing milled cheese with twice its weight of sand, the mixture was wrapped in a tightly woven cloth and the liquid squeezed out by a cylindrical press. This extract was used directly for a coagulation test after removal of fat by centrifugation. They reported that residual rennin remained active in cheese for at least 8 months. The difference in coagulating power of extracts from Swiss and Cheddar cheese was attributed to the difference in cooking temperatures. However, no quantitative data were presented. Peterson et al. (60, 61) also reported the presence of residual rennin in cheese. They used a cheese slurry for hydrolysis of a standard casein suspension and reported their results in terms of a cheese protease unit which was defined as the amount of enzyme which, when diluted to 10.2 ml and incubated for 5 hours under standard conditions, will liberate 1 mg of non-protein nitrogen. A protease activated by addition of cysteine to a cheese slurry at pH 5.0 was considered as endocellular bacterial protease, but they could not differentiate extracellular bacterial protease from rennin. However, they did not study the effect of cysteine on the pure enzymes.

Residual coagulants in whey and curd, and the distribution of coagulating enzymes between whey and curd has not been reported and evaluated with respect to their importance to the cheese industry.

Killing (39) demonstrated that cooking the cheese curd at 55 C for 55 minutes did not entirely destroy the coagulation power of rennin in whey. He concluded that some enzyme must remain active in cheese which was cooked below 55 C. Therefore, cheese such as Cheddar, Colby, Edam, Gonda, Monterey, Brick, Swiss, Parmesan and others which are usually cooked at 45-50 C must contain some residual rennin, the amount present being dependent upon exact time and temperature of cooking.

Measuring Rennin Activity

Many tests have been suggested for measuring rennin activity, but an ideal test which can measure the activity precisely has never been devised. Most of the tests are based on measurement of the length of time required for a given amount of milk substrate to clot after addition of a known diluted rennin solution.

The rennin activity of an unknown sample is determined by comparing its activity with that of a standard rennin solution. The standard is usually arbitrarily assigned an activity value of 100 rennin units (RU) per milliliter standard rennin solutions are stored at low temperatures and renewed frequently to maintain their activity value.

Ernstrom (20) stated that the lack of a uniform standard for rennin activity in different laboratories had made it difficult for research workers to compare their results even though enough care was taken to maintain the strength of standard rennin.

Methods for determination of rennin strength can be divided in three major groups:

I. Those which measure the change in viscosity of the substrate (40, 73, 74).

II. Those which are based on visual observation of precipitation or coagulation of the substrate (7, 8, 14, 70, 80, 84).

III. Those which measure proteolysis by rennin (10, 46, 60).

The essential difference between the first and second method is only in the means of detecting the coagulation end point (14, 21, 80, 84).

The early findings of Graber (28) using milk as a substrate for rennet activity studies indicated that the coagulation power of rennet was influenced by the composition of the milk, particularly with respect to calcium salts. He also reported that it was affected by the temperature of the milk before and during the test. He used 2 quarts of milk for samples and checked the coagulation by tipping the container to one side. By this method it was very difficult to detect the coagulation end point. Therefore, to eliminate these difficulties and the large waste of milk, Sommer and Matsen (80) developed an apparatus which consisted of a water bath in which 125 ml wide-mouth test bottles containing 50 ml milk substrate rotated slowly under direct observation of the

operator. The coagulation time was measured by a separate timing device in terms of counts (at 16 counts per minute) which was started when the enzyme was added to the milk or substrate, and stopped by the operator when coagulation became visible. The same machine is still used by the rennet industry with few modifications.

Since milk varies from cow to cow and species to species, rennet strength cannot be compared on different milk samples. Therefore, to eliminate these difficulties, Berridge (7) developed a substrate for a coagulation test in which 60 g nonfat dry milk (NDM) were reconstituted in 500 ml 0.01 M $CaCl_2$. He suggested warming the substrate to 30 $\pm$ 0.2 C for 30-35 min before using. This substrate gave more reproducible results than milk, and was less subject to the many factors that caused variation in clotting time of normal milk. Ernstrom (16) found that the clotting time of Berridge's substrate continued to increase for about 20 hours during storage at 2 C following reconstitution. He suggested that Berridge's substrate should be stored at 2 C for 20 hours in order to achieve constant coagulation time. Ernstrom (18) used the apparatus of Sommer and Matsen (80) and standardized a test procedure to measure the milk clotting activity of rennin in which 25 ml of well mixed substrate were pipetted at 2 C into 125 ml wide-mouth test bottles. The test bottles were placed on the apparatus at 30 C for about 20 min to allow the substrate to come to temperature. One ml of a diluted rennin sample was pipetted into each of two test bottles. As soon as the rennin was released from the pipette, the corresponding counter on the apparatus was started. The pipette was allowed

to drain by touching the tip against the inside surface of the bottle just above the substrate. The sample was swirled gently to insure complete mixing and replaced on the test apparatus. The first appearance of visible flakes on the inner glass surface was taken as the end point. The rennin activity of an unknown sample was measured by comparing its coagulation time with that of standard rennin tested simultaneously, and taking their dilutions into consideration. For quantitative expression in terms of rennin units he suggested the formula,

$$RU/ml = 100 \frac{Ts}{Tu} \times \frac{Cs}{Cu}$$

where RU/ml = rennin unit per ml of unknown,

Ts = coagulation time of the standard,

Tu = coagulation time of the unknown,

Cs = concentration of the standard,

Cu = concentration of the unknown.

DeMan and Batra (14) used an automatic blood-clot timer for measuring the rennet clotting time of milk, skim milk, homogenized milk and 10 per cent fat cream. Storrs (84) developed an automatic tester to determine the clotting activity of rennet. He used the substrate prepared by reconstituting NDM and aging for 2 hours at 37 C before use. However, he has not reported the quantity of NDM and water used.

Gorini and Lanzavecchia (27) described a substrate for measuring the milk clotting activity of proteolytic enzymes. Their substrate was buffered at

pH 5.8 and consisted of 1 g NDM in a mixture of 70 ml, 6.6×10^{-2} M cacodylic acid, 30 ml 6.6×10^{-2} M triethanol amine and 1 ml 3 M $CaCl_2$. This substrate gave a very sharp end point.

Everson and Winder (21) used an instrument for measuring the sound velocity in liquids for the determination of rennet coagulation time automatically. At coagulation, an increase in sound velocity occurred which was recorded as the end point. They used a modified buffered substrate containing 15 g NDM, 0.0699 g $CaCl_2$, 61.5 ml 0.066 M cacodylic acid and 26.3 ml; 0.066 M triethanol amine per 100 ml of substrate.

An agar diffusion slide assay using a thin layer of calcium caseinate has been developed by Lawrence and Sanderson (46) as a micromethod for quantitative estimation of rennet and other proteolytic enzymes in very low concentration.

Peterson et al. (61) measured cheese protease by hydrolysis of standard casein suspensions with cheese slurries. The casein suspension was prepared by combining 10 g purified casein with 25 ml 1 N NaOH, 40 ml 0.2 M sodium citrate, 2 g gum-ghatti and distilled water to a volume of 400 ml. Concentrated H_2SO_4 was added to adjust the pH to 5.0. The mixture was blended for 24 minutes and homogenized 3-4 times through a hand homogenizer.

Cheese samples for protease analysis were prepared by combining 20 g cheese with 180 ml distilled water and homogenizing 5 to 6 times.

For protease analysis, 1 ml 1 M acetate buffer at pH 5.2, 1 ml 0.015 M cysteine hydrochloride and 0.2 ml toluol were added to 5 ml casein

suspension and shaken for 2 min. This mixture and cheese slurry were tempered to 40 C, and from 0.5 to 3 ml (depending upon the activity) of the cheese slurry were added to the above mixture and the total volume made to 10.2 ml with distilled water. The mixture was shaken and homogenized 2 to 3 times. After removal of 1 ml sample for determination of initial nonprotein nitrogen, it was incubated to 40 C for 5 hours. At the end, the mixture was rehomogenized and 1 ml sample was taken for final nonprotein nitrogen determination. The net nonprotein nitrogen was used as a measure of protease activity in cheese. However, this method was very time consuming.

A rennin-sensitive substrate was developed by Wang (90) to measure the very low concentrations of rennin in cheese. It was a modification of Gorini and Lanzavechhia (27) substrate and consisted of 6 g low-heat NDM in 500 ml buffer containing 50 ml 0.5 M cacodylic acid, 50 ml 0.2 M $CaCl_2$, 30 ml 0.2 M triethanol amine and 370 ml distilled water. He stored the substrate at 2-4 C for 20 hours before use. He obtained reproducible results on this substrate when used for determination of rennin activity in very low concentrations. This substrate was found to be 20 times more sensitive than Berridge's substrate. The method described was simple and short.

Reyes (68) developed a procedure to measure very low concentrations of rennin from curd obtained from freshly coagulated milk. He made 1:15 curd-water slurries and adjusted them to pH 6.8. The slurry filterate was used to measure activity.

EXPERIMENTAL METHODS

Measuring Clotting Activity

The activity of an enzyme from Endothia parasitica[1] (EP enzyme) was measured on a special substrate proposed by Wang (90). The substrate was prepared by combining 50 ml 0.5 M cacodylic acid, 50 ml 0.2 M $CaCl_2$, 30 ml 0.2 M triethanol amine, 370 ml double distilled water and 6 g low heat non-fat dry milk (NDM). The substrate was stored at 2 C for 20 hours to ensure equilibrium. The final pH of the substrate was 5.8.

Measurement of coagulation time was carried out on an apparatus similar to one described by Sommer and Matsen (80) on which the test bottles rotated at 8 rpm. Twenty-five milliliters of substrate were pipetted at 2 C into a 125 ml wide-mouth test bottle. The substrate was tempered for 20-30 min in the tester at 30 C prior to addition of 2 ml of diluted enzyme solution. The time between addition of the enzyme and appearance of coagulated flakes in the substrate was taken as the coagulation time. Unlike milk substrate used for measuring coagulation time, this substrate did not form a film on the inner side of the test bottle. The coagulation end point was recorded when flakes appeared in the body of the substrate.

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[1] Supplied by Chas. Pfizer and Co., Inc., Milwaukee, Wisconsin.

A standard rennet extract[1] assigned an arbitrary activity value of 100 rennin units (RU)/ml was used as a reference for all activity measurements. The standard rennet had an activity such that 1 ml of a 1:5000 dilution induced coagulation in 25 ml substrate in 4.75 $\pm$ 0.25 minutes at 30 C. A reference EP enzyme solution was also prepared by dissolving the dry enzyme preparation in water to a concentration such that its activity was equivalent to that of the standard rennet when tested on the special substrate.

Whenever clotting activities of whey, curd slurries or curd slurry supernatants were measured, a reference standard of the EP enzyme solution was adjusted by dilution to give coagulation times within $\pm$ 5 per cent of the unknown solutions. Activities of EP enzyme solutions were reported in terms of rennin units (RU) since they were related to standard having the same activities on the test substrate at pH 5.8.

Enzyme activity was calculated as follows (19):

$$RU/ml = \frac{Ts}{Tu} \times \frac{Du}{Ds} SA$$

where RU = Rennin units per ml of unknown,

Ts = coagulation time of the standard,

Tu = coagulation time of the unknown,

Ds = dilution of the standard,

Du = dilution of the unknown,

SA = rennin units per ml of undiluted standard.

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[1]Supplied by Dairyland Food Laboratories, Inc., Waukesha, Wisconsin.

Preparation of Curd and Whey

Fresh raw milk obtained from Utah State University Dairy Products Laboratory was pasteurized at 63 C for 30 minutes, cooled to 2-4 C and stored for 12 hours to eliminate cool-aging differences. The cold milk was quickly warmed to 25 C in a 50-60 C water-bath, and the pH adjusted to the desired level by addition of 1 N HCl or 0.5 N NaOH. It was then divided into samples of desired weight and warmed to 30 C in a water bath. Measured volumes of EP enzyme solutions were added to each sample of milk (6.72 RU per 454 g milk) and the sample poured immediately into a 250 ml centrifuge bottle which was held at 30 C for 15 minutes after appearance of the first signs of coagulation. A fresh dilution of coagulating enzymes was made each day, and stored in the refrigerator during use. The curd was broken with a spatula, and the bottles were shaken well to break the curd completely. The sample was centrifuged for 20 minutes at 5000 G in an International Centrifuge to separate the curd and whey. The clear whey was decanted into a beaker and weighed. The weight of the curd was obtained by difference. The weight of the coagulants was considered part of the sample weight. The whey was stored in the refrigerator until its enzyme activity was measured.

Curd slurries were prepared by the method suggested by Wang (90). The slurries were made in various ratios of curd to water, and when necessary the pH was adjusted to release the enzyme from the curd. The quantity of

HCl or NaOH added for pH adjustment was counted as part of the water in the slurry.

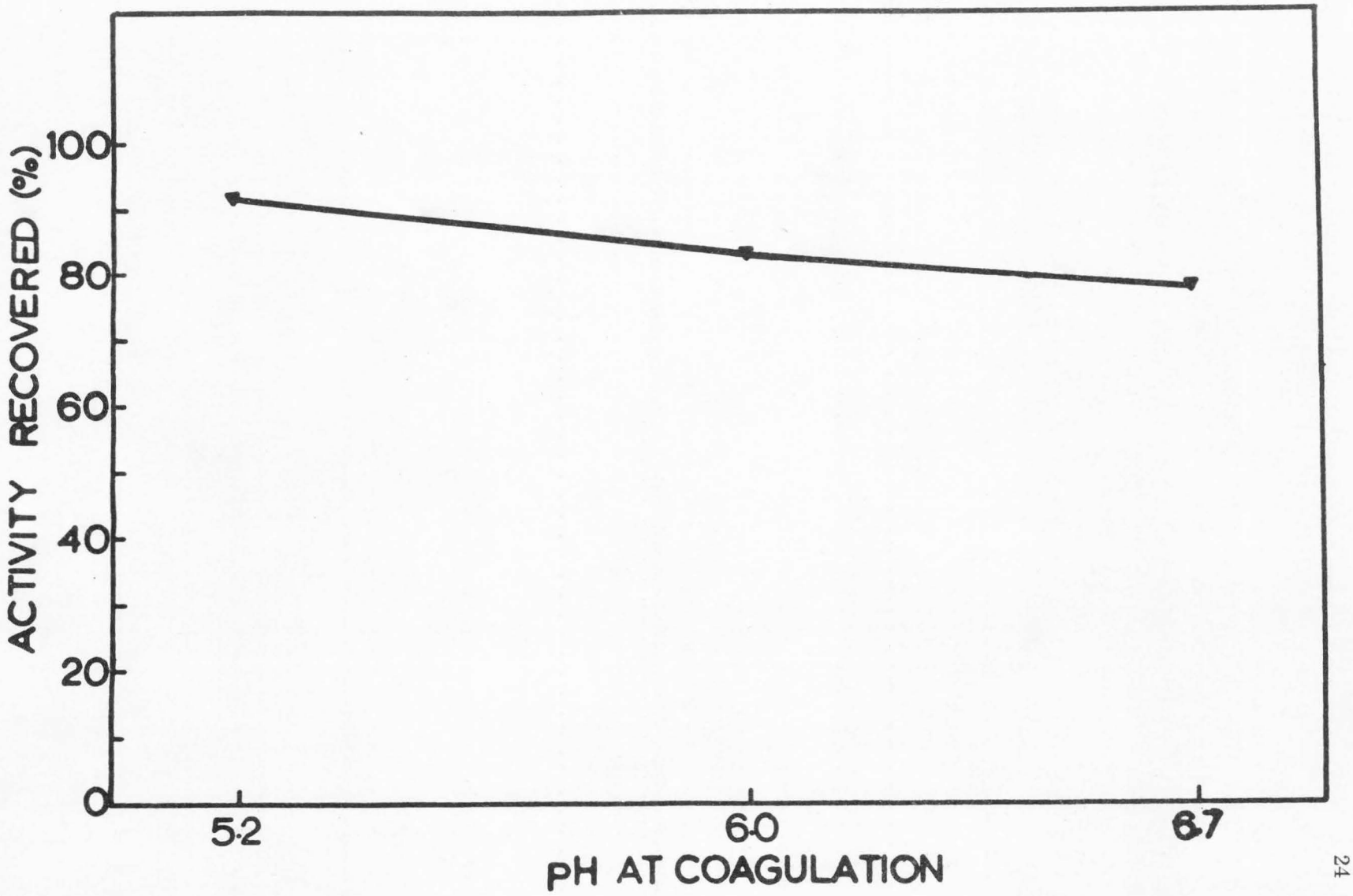
Slurries were held in the refrigerator until assayed (90) to prevent the loss of enzyme activity. Before testing, slurries were mixed well to obtain homogeneous samples, and filtered through grade 637 coarse filter paper. The filtrate was used for measuring enzyme activity in curd (68 , 69).

RESULTS

Measurement of EP Enzyme Activity in Freshly Coagulated Milk

Pasteurized (63 C for 30 minutes) whole milk was warmed to 25 C, and divided into three lots which were adjusted to pH 5.2, 6.0 and 6.7, respectively. Each lot was subdivided into 454 g samples which were then warmed to 30 C and set with 6.72 RU of EP enzyme. The milk was held undisturbed for 15 minutes after the appearance of the first sign of coagulation. The first sign of coagulation appeared in 3-4 minutes at pH 5.2, 11-13 minutes at pH 6.0 and 35-40 minutes at pH 6.7 when set with rennin. When set with EP enzymes the first sign of coagulation appeared after 2-3 minutes, 10-12 minutes and 30-35 minutes at pH 5.2, 6.0 and 6.7, respectively. The coagulated milk was transferred to a Waring blendor and mixed at high speed for 2 minutes to form a smooth slurry. The slurry was allowed to stand at 25 C for 45-60 minutes. The milk fat which churned during blending was removed from the surface of the mixture and weighed. The weight of the fat was subtracted from the original weight of the milk and the well-mixed defatted slurry tested for clotting activity. The activity per 2 ml (2.0235 g) was used to calculate total activity in the slurry which was expressed as per cent of the activity added to the milk. Results are presented in Figure 1, where each point for EP enzyme represents a mean value of 6 or 10 replications. Standard deviations for

Figure 1. Effect of pH on per cent of original Endothia parasitica (EP enzyme) enzyme activity detected in freshly coagulated milk.


ACTIVITY RECOVERED (%)
100
80
60
40
20
0
5·2
6·0
6·7
pH AT COAGULATION

per cent EP enzyme recovered at pH 5.2, 6.0 and 6.7 were ± 1.36, ± 1.05 and ± 0.82 per cent (see Appendix, Tables 3, 4, and 5).

Heat Inactivation of EP Enzyme in Whey at pH 5.20

One pound of pasteurized milk was adjusted to pH 5.20 with 1 M HCl and warmed to 30 C. The milk was set with 26.8 RU of EP enzyme and allowed to stand for 15 minutes after the first sign of coagulation. The clot was broken with a spatula and the whey was separated from the curd by centrifugation. The whey was divided into four 50 ml samples and heated for 5 minutes at temperatures of 25, 50, 63, 68 and 73 C, respectively. Each sample was cooled to 25 C and tested for survival of EP enzyme activity. Results are presented in Table 1.

Table 1. Estimate of percentage of original EP enzyme activity remaining in whey at pH 5.20 following 5 minutes heat treatments at various temperatures

Temperature	Coagulation time	Enzyme activity
(°C)	(Average counts)	(%)
25	12.3	100.0
50	59.3	20.24
63	21741	0.05
68	No coagulation	-----
73	No coagulation	-----

The per cent of original EP enzyme 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.

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

The whey heated to 63 C failed to clot the substrate in 18.1 hours.

Effect of pH on the Distribution of EP Enzyme between Curd and Whey

Curd and whey obtained from milk set at pH 5.2, 6.0 and 6.7 with EP enzyme were separately weighed, and a 1:5 curd-water slurry was prepared for enzyme analysis. pH values of the slurries were 5.6, 6.4 and 6.9 when prepared from curd at pH 5.2, 6.0 and 6.7. The increase of pH was attributed to colloidal calcium phosphate present in the curd which dissolved upon preparation of the slurry. Slurries were held at 2-4 C for 2 hours after which 2 ml (2.0235 g) of well-mixed slurry and 2 ml (2.0268 g) of whey were tested for clotting activity. Measured activities were used to calculate the

total activity in the curd and whey. The pH at which the milk was set affected the relative amount of curd and whey recovered from each sample of milk. This is shown in Table 2 along with the measured activity in the curd and whey. The sum of activities measured in the whey and curd was expressed as per cent of the activity added to the milk. Results are shown in Figure 2 where each bar represents the mean value of four replications.

Distribution of EP enzyme between whey and curd, and the total recovery of EP enzyme in the curd were both affected by the pH at which the curd was formed.

Effect of pH Adjustment of Slurry on the Recovery of EP Enzyme and Enzyme Activity from Curd

Coagulated particles of milk added to the substrate as part of the slurry made it difficult to identify the coagulation end point. Considerable practice was required before the operator could distinguish between coagulated particles formed by the substrate and coagulated curd particles added with the slurry. The test would be much improved if curd particles could be separated from the sample before assay without interferring with enzyme concentration. This was attempted by using centrifuged curd-slurry supernatants in place of complete slurries.

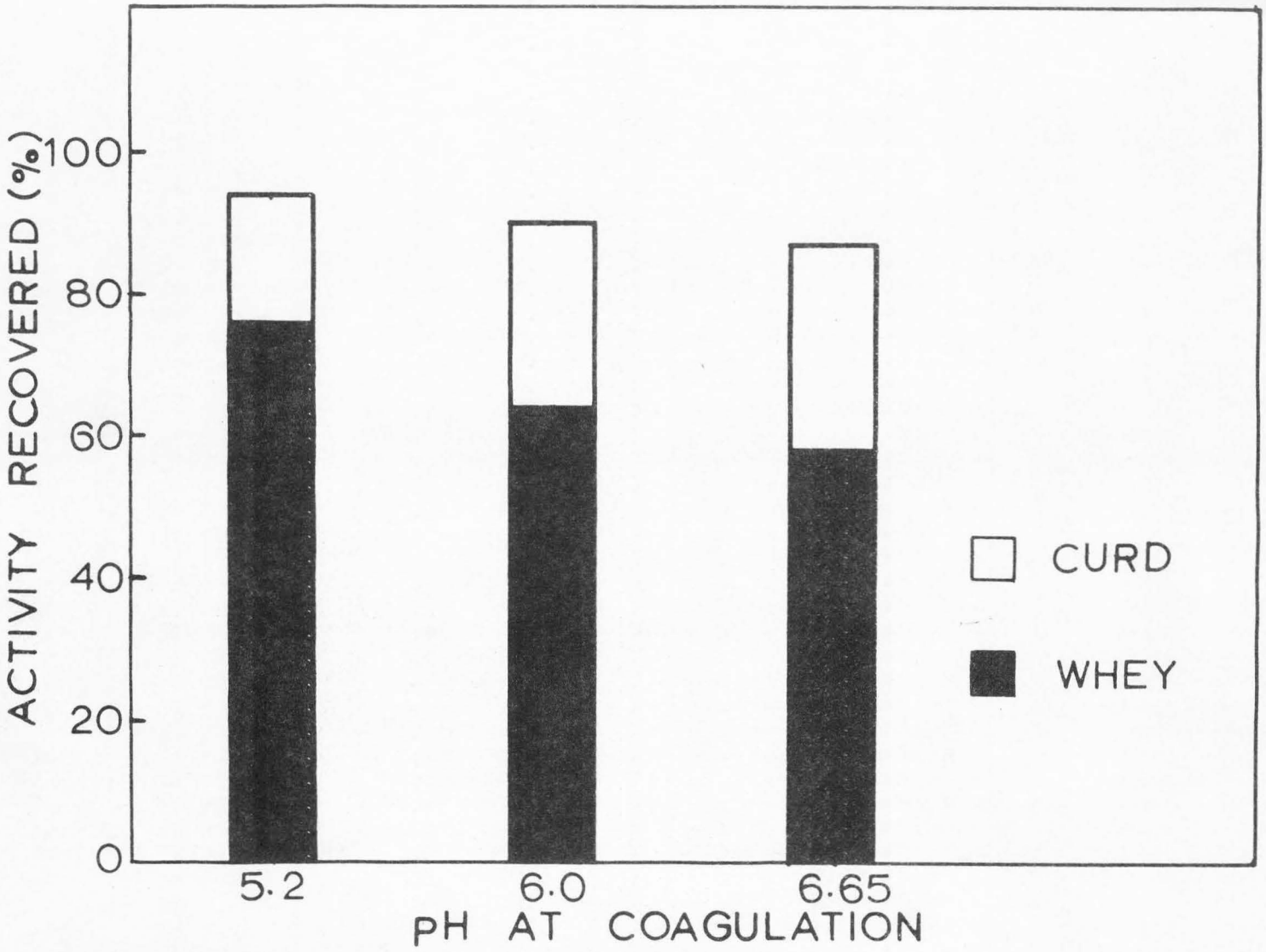
Furthermore, if this test is to be used for measuring enzyme activity in cheese curd it will be necessary to quantitatively assay the enzyme activity

Table 2. Effect of pH of coagulation on the amount[a] of curd and whey obtained from 454 g of milk set with EP enzyme and the clotting activity measured in curd and whey

pH of milk	Quantity		Activity measured in whey	Activity measured in curd	Total activity measured whey + curd	Per cent of theoretical activity measured in curd
	Whey g	Curd g				
5.20	386.4	79.4	5.12	1.28	95.20	90.17
6.0	359.5	106.5	4.32	1.71	89.44	71.07
6.70	342.40	123.60	3.93	1.95	87.53	70.01

[a]Average values (see Appendix, Tables 6, 7, and 8).

Figure 2. Effect of pH on the recovery of EP enzyme in the curd and whey separated from freshly coagulated milk.


ACTIVITY RECOVERED (%)
100
80
60
40
20
0
5.2
6.0
6.65
PH AT COAGULATION
CURD
WHEY

in curd which has a pH value approximating 5.2. This appeared quite feasible with EP enzyme.

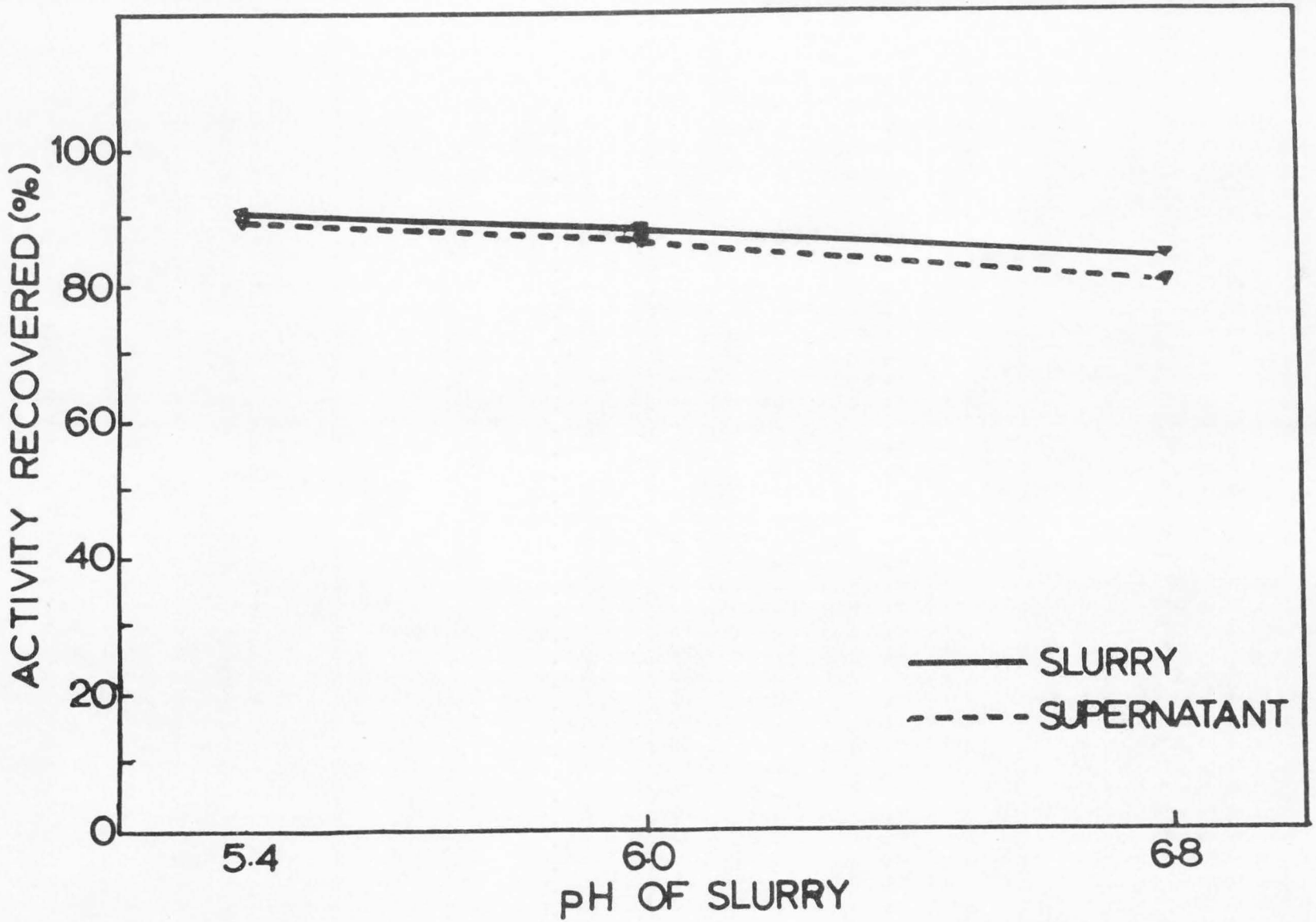
Whey and curd were separated from one lot of milk coagulated at pH 5.2. A 1:5 curd-water slurry was prepared and divided into three samples which were then adjusted to pH 5.4, 6.0 and 6.8, respectively, and held at 2-4 C for 2 hours. Each slurry was tested for activity. Activity in the whey which was separated from the curd at pH 5.2 was measured and subtracted from the amount of activity added to the milk (6.72 RU). The difference was assumed to be the theoretical activity left in the curd. Slurries were then centrifuged to obtain a clear supernatant liquid which was tested for activity and compared with that of the slurry.

The clotting activity of EP enzyme obtained from slurries and supernatants were expressed as a per cent of the theoretical activity in the curd. The results are presented in Figure 2 where each point represents a mean value of 4 replications. Standard deviations for per cent recovery of activity in curd varied from $\pm$ 0.05 to $\pm$ 6.77 per cent (see Appendix, Tables 9 and 10).

Effect of Dilution of pH-adjusted Slurries on the Recovery of EP Enzyme from Curd

Curd and whey were separated from milk coagulated at pH 5.2. The whey was tested for activity which was subtracted from the activity added to the milk in order to arrive at theoretical activity remaining in curd. Carefully weighed portions of the well-mixed curd were used to make 1:5, 1:7, 1:11

Figure 3. Effect of pH of 1:5 curd-water slurries on the per cent recovery of EP enzyme from curd formed at pH 5.2.


ACTIVITY RECOVERED (%)
100
80
60
40
20
0
5.4
6.0
6.8
pH OF SLURRY
SLURRY
SUPERNATANT

and 1:15 curd-water slurries. The pH values were adjusted to 5.4. Slurries were held at 25 C for 30 minutes before being tested. Part of each slurry was filtered through No. 1 filter paper to obtain a clear supernatant for enzyme analysis as suggested by Reyes (68) instead of centrifuging the slurries.

The EP enzyme activities that were measured in the slurry filterates were expressed as per cent of the theoretical activities in curd. Results are shown in Figure 4 where each point represents the average values of four replications. Standard deviations for per cent recovery varied from $\pm$ 2.40 to $\pm$ 3.06 per cent (see Appendix, Table 11).

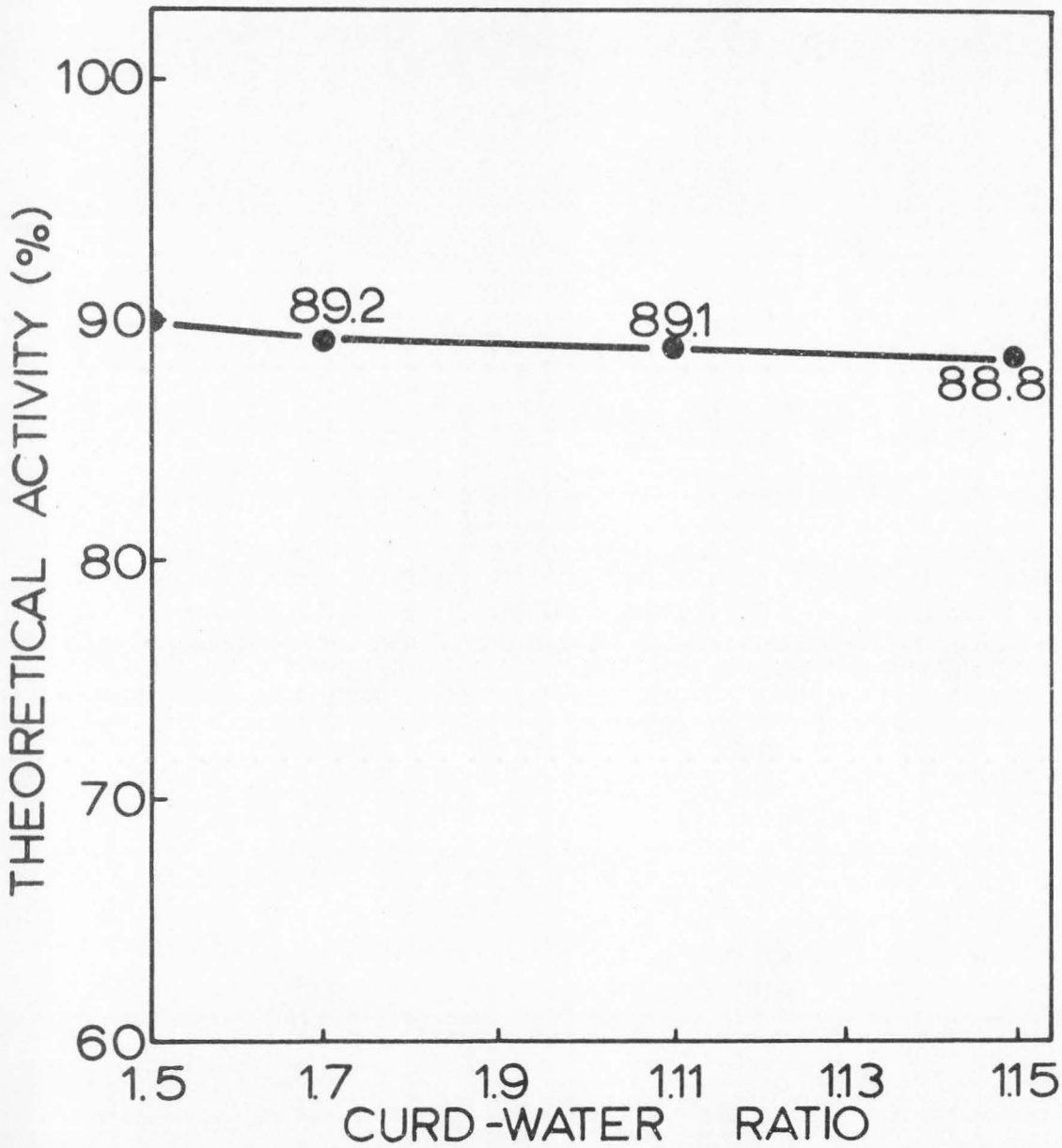
Dilution of the slurries at pH 5.4 had no effect on the recovery of EP enzyme from slurry filterates, but had an adverse effect on activity of enzyme as dilution was increased.

Two milliliters of slurry filterate from EP enzyme slurry prepared at a 1:5 curd-water ratio and adjusted to pH 5.4, produced substrate coagulation in approximately 35-40 minutes.

Effect of NaCl on the Measurement of EP Enzyme Activity in Curd

Since curd at pH 5.2 is soluble at low NaCl concentrations (87) the proper concentration of salt might assist the release of rennin and EP enzyme from curd. Wang (90) suggested that 1.67 per cent NaCl was needed to get complete recovery of rennin from cheese curd.

Figure 4. Effect of curd-water ratio of slurry at pH 5.4 on the per cent recovery of EP enzyme in curd formed at pH 5.2.

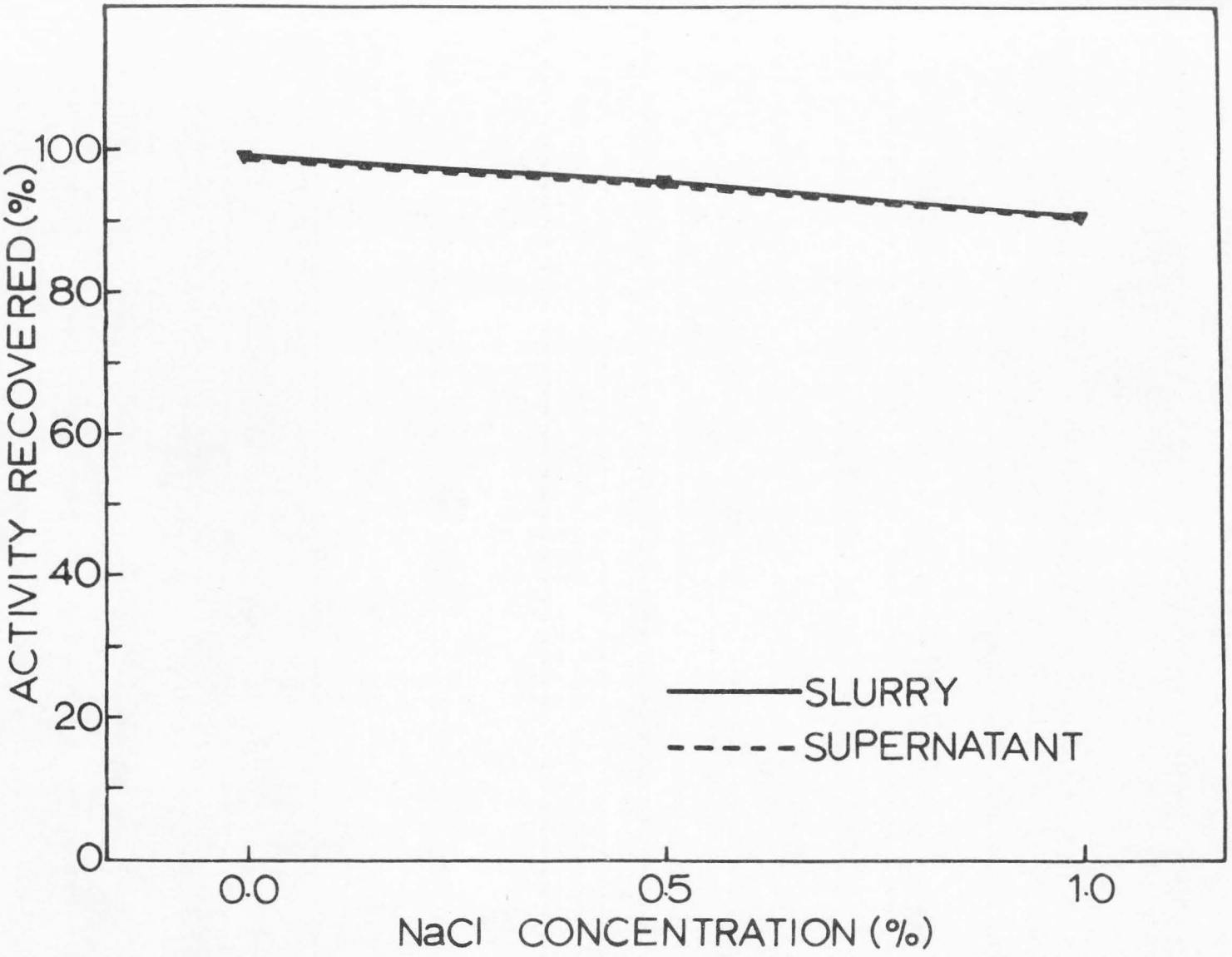

100
90
80
70
60
THEORETICAL ACTIVITY (%)
89.2
89.1
88.8
1.5
1.7
1.9
1.11
1.13
1.15
CURD-WATER RATIO

Endothia parasitica enzyme was used to prepare curd and whey from a single lot of milk at pH 5.2. Slurries (1:5 curd-water) were prepared, and the pH adjusted to 5.4 in the slurry. Each slurry was divided into three lots, and NaCl was added to concentrations of 0.0, 0.5 and 1.0 per cent, respectively. They were then held at 2-4 C for 2 hours. Part of each slurry was centrifuged to obtain a clear supernatant. The slurries and slurry supernatants were tested for enzyme activity. The activity of each whey sample was measured and subtracted from the amount added to the milk to arrive at the theoretical amount of enzyme activity in the curd.

Clotting activities of EP enzyme recovered in slurries and slurry supernatants were expressed as a per cent of the theoretical activity in the curd. The results are presented in Figure 5 where each point represents an average of six replications. Standard deviations for the per cent recovery varied from $\pm$ 0.23 to $\pm$ 1.02 per cent (see Appendix, Tables 12 and 13).

Sodium chloride inhibited milk clotting activity at all concentrations. The same effect was observed when proportional amounts of NaCl were added to the substrates prior to testing with standard rennin. This suggested that the sodium ions might have interferred with the coagulation of the casein in milk. Maze (53) reported that NaCl prolonged renneting time in the milk due to partial replacement of calcium from casein molecules by sodium ions. However, it could also serve to dilute out the effect of the free soluble calcium ions which are essential to the coagulation of paracasein.

Figure 5. Effect of NaCl in 1:5 curd-water slurries on the per cent EP enzyme accounted for in curd formed at pH 5.2 (EP enzyme slurries adjusted to pH 5.4).


ACTIVITY RECOVERED (%)
100
80
60
40
20
0
0·0
0·5
1·0
NaCl CONCENTRATION (%)
SLURRY
SUPERNATANT

DISCUSSION

When milk-coagulating enzymes are used in cheese manufacture, some enzyme activity remains in the cheese (90), some is lost in the whey and some is probably destroyed during cheese making. Any attempt to quantitatively account for the distribution and fate of these coagulants would require a test procedure capable of measuring all the residual enzyme in the curd as well as the whey. Since enzyme activity losses during cheese making could be substantial, recovery procedures were developed on curd and whey separated from milk freshly coagulated by EP enzyme at 30 C. No heating of the curd or whey was employed. This resulted in curd much higher in moisture than one would expect from most cheese making operations, but it was essential that the enzyme remain stable in order to evaluate the procedure.

Concentrations of EP enzyme was employed which approximated those used in cheese making.

The very sensitive substrate proposed by Gorini and Lonaavacchia (27) and modified by Wang (90) was satisfactory for measuring coagulation produced by this enzyme at concentrations recovered in curd and whey.

No special preparation of the sample was required for measuring enzyme activity in whey. A measured amount of whey (2 ml) was added to 25 ml of substrate, and the coagulation time measured and compared with that produced in a duplicate substrate by a known dilution of a standard enzyme solution. The standard EP enzyme was diluted with heated whey so that the

conditions remained the same except for enzyme content in both the whey samples. The end point of the coagulation was indicated by the appearance of tiny flakes in the body of the substrate.

Measurement of enzyme activity in curd was more complicated. When curd-water slurries were prepared, fine curd particles in the slurry obscured the coagulation end point. This problem was solved by centrifuging the slurry and using the supernatant for inoculating the substrate. However, under certain conditions the enzyme was associated with the curd in such a way that its activities were only partially measurable.

Most of the EP enzyme was released into curd slurry supernatants in 1:5 curd-water slurries at pH 5.4. At pH 6.0 and 6.8, there was progressively less EP enzyme available for assay. However, even at pH 6.8, 80 per cent of the EP enzyme in the curd was measurable in the supernatant.

The effect of pH on the affinity of curd for EP enzyme was also reflected in the distribution of this enzyme between curd and whey when milk was coagulated at various pH values. At pH 6.7, 70.01 g of curd contained 42 per cent of the EP enzyme added to 454 g of milk. At pH 5.2, 90.17 g of curd contained 24 per cent of the activity in the same amount of milk.

The curd:water ratio had a great effect on the recovery of enzyme from curd. Since at pH 5.2 very little EP enzyme remained in curd, a curd-water ratio of 1:5 was most effective for enzyme recovery. More than 1:5 curd-water ratio had adverse effects on recovery because the slurries became

more and more dilute and blending apparently had a destructive effect on enzyme released from the curd (68).

Release of rennin from curd by adding 1.67 per cent NaCl as proposed by Wang (90) was unsuccessful because any amount of salt added to the substrate or added with the enzyme delayed coagulation of the substrate. The same effect was observed with EP enzyme when salt was added to the slurry.

Recommended Procedure for Measuring EP Enzyme Activity in Whey

1. Prepare a substrate by combining 50 ml 0.5 M cacodylic acid, 50 ml 0.2 M $CaCl_2$, 30 ml 0.2 M triethanol amine and dilute to 500 ml with distilled water and 6 g NDM. Store at 2 C for 20 hours before using.

2. Cool whey sample rapidly and store under refrigeration until tested.

3. Heat a portion of the whey to be tested to 63 C for 10 minutes and cool to 25 C with cold water. Use this whey to make appropriate dilution of a standard EP enzyme solution.

4. Transfer 25 ml of substrate to 125 ml wide-mouth test bottle and temper to 30 C in the tester for 20-30 minutes.

5. Add 2 ml whey to the substrate and start the timer at the instant the whey is released from the pipette.

6. Stop the timer when the first sign of coagulation appears in the substrate.

7. An enzyme solution of known concentration must be diluted such that 2 ml of the standard will give approximately the same coagulation time when run simultaneously alongside the unknown whey sample.

8. Calculate the enzyme activity in the whey with the following formula.

$$RU/ml = \frac{Ts}{Tu} \times \frac{Du}{Ds} \times SA$$

where RU/ml = rennin units per ml of unknown whey sample,

TS = coagulation time of the standard,

Tu = coagulation time of the unknown whey,

Ds = dilution of the standard,

Du = dilution of the whey,

SA = rennin units per ml of undiluted standard.

Recommended Procedure for Measuring EP Enzyme Activity in Curd

1. Accurately weigh 80 g EP enzyme curd, transfer to a Waring blendor and add 150 ml distilled water at 35 C. Blend at high speed for 1 minute. Add 250 ml distilled water at 35 C and blend for 1 additional minute or until the slurry is smooth. Adjust the pH to 5.4 with 1 N HCl and store at 2 C for 2 hours.

2. Make appropriate dilution of standard EP enzyme with distilled water.

3. Allow the curd slurry to stand at 25 C for 30 minutes. Filter a small portion of the curd slurry to obtain curd-free filtrate.

4. Add 2 ml of the supernatant to 25 ml substrate and test for activity as described in steps 4-7 under the procedure for measuring the activity in whey. This will give the activity per ml (1.0025) of slurry.

5. Dilution of the curd including the addition of HCl must be included in calculating dilution (of unknown).

The test procedures outlined here gave quantitative recovery of added EP enzyme from curd and whey separated from freshly coagulated milk at pH 5.2. It is recognized that the characteristics of cheese curd are different from that of the curd used in these experiments. However, the principles demonstrated here must be taken into consideration when measuring the rennin or EP enzyme content of cheese curd.

The sensitivity of the test might be improved if the buffer concentration in the substrate were increased. This would make it possible to use more inoculum than was used in these experiments.

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APPENDIX

Table 3. Clotting activity of EP enzyme recovered from freshly coagulated milk at pH 5.2

Replication	Activity added	Activity recovered	Recovery
	(RU)	(RU)	(%)
1	6.72	6.20	92.26
2	6.72	6.17	91.98
3	6.72	6.03	89.73
4	6.72	6.10	90.77
Mean	6.72	6.12	91.19
SD		±0.06	±1.36

Table 4. Clotting activity of EP enzyme recovered from freshly coagulated milk at pH 6.0

Replication	Activity added	Activity recovered	Recovery
	(RU)	(RU)	(%)
1	6.72	5.55	82.51
2	6.72	5.51	81.94
3	6.72	5.45	80.46
4	6.72	5.48	81.57
5	6.72	5.45	81.11
6	6.72	5.66	84.22
7	6.72	5.61	83.48
8	6.72	5.59	83.19
9	6.72	5.53	82.23
10	6.72	5.52	82.14
Mean	6.72	5.54	82.29
SD		±0.07	±1.05

Table 5. Clotting activity of EP enzyme recovered from freshly coagulated milk at pH 6.7

Replication	Activity added	Activity recovered	Recovered
	(RU)	(RU)	(%)
1	6.72	5.27	78.52
2	6.72	5.31	78.96
3	6.72	5.33	79.41
4	6.72	5.25	78.09
5	6.72	5.22	77.67
6	6.72	5.24	78.01
7	6.72	5.21	77.52
8	6.72	5.19	77.23
9	6.72	5.36	79.76
10	6.72	5.26	78.23
Mean	6.72	5.26	78.34
SD		±0.05	±0.82

Table 6. Effect of pH of coagulation on the curd and whey obtained from 454 g milk set with EP enzyme[a] at pH 5.2 and on the clotting activity recovered in the curd and whey

Replication	Quantity		Activity measured in whey	Theoretical activity in curd	Activity measured in curd	
	Whey	Curd			Total	%
1	390.1	75.9	5.10	1.34	1.24	92.50
2	384.1	81.4	5.07	1.37	1.19	86.80
3	386.2	79.8	5.12	1.32	1.16	87.80
4	385.4	80.6	5.18	1.26	1.18	93.60
Mean	386.4	79.4	5.12	1.32	1.16	90.17
SD			±0.06	±0.03	±0.03	±4.12

[a] 6.72 RU of EP enzyme.

Table 7. Effect of pH of coagulation on the curd and whey obtained from 454 g milk set with EP enzyme[a] at pH 6.0 and on the clotting activity recovered in the curd and whey

Replications	Quantity		Activity measured in whey	Theoretical activity in curd	Activity measured in curd	
	Whey	Curd			Total	%
1	360.8	105.4	4.34	2.38	1.62	68.06
2	358.2	107.8	4.21	2.51	1.85	73.70
3	361.8	104.2	4.48	2.24	1.59	70.98
4	357.3	108.7	4.26	2.46	1.76	71.54
Mean	359.5	106.5	4.32	2.40	1.71	71.07
SD			±0.02	±0.014	±0.015	±5.44

[a] 6.72 RU of EP enzyme.

Table 8. Effect of pH of coagulation on the curd and whey obtained from 454 g milk set with EP enzyme[a] at pH 6.7 and on the clotting activity recovered in the curd and whey

Replications	Quantity		Activity measured in whey	Theoretical activity in curd	Activity measured in curd	
	Whey	Curd			Total	%
1	345.6	120.4	3.95	2.77	1.95	70.39
2	340.2	125.8	4.10	2.62	1.90	72.51
3	341.1	124.9	3.85	2.87	1.94	67.59
4	342.8	123.3	3.83	2.90	2.01	69.55
Mean	342.40	123.6	3.93	2.79	1.95	70.01
SD			±0.015	±0.015	±0.002	±4.15

[a] 6.72 RU of EP enzyme.

Table 9. Effect of pH on slurry on the recovery of EP enzyme[a] from curd formed at pH 5.2 (test performed on slurry)

Replication	Activity recovered in whey	Theoretical activity in curd	Activity recovered in slurry[b]					
			pH 5.4		pH 6.0		pH 6.8	
	(RU)	(RU)	(RU)	(%)	(RU)	(%)	(RU)	(%)
1	20.31	6.57	5.76	87.67	2.58	86.87	2.48	83.50
2	20.26	6.62	6.07	91.69	2.60	86.37	2.56	85.04
3	20.41	6.47	5.78	89.33	2.61	89.69	2.49	86.56
4	20.79	6.09	5.70	93.59	2.63	87.66	2.58	87.00
5	10.46	2.98	----	-----	2.55	85.57	2.45	82.21
6	10.56	2.88	----	-----	2.58	89.58	2.41	83.68
7	10.40	3.04	----	-----	2.63	86.51	2.56	84.21
8	----	----	----	-----	2.65	88.62	2.51	83.94
Mean	20.44	6.44	5.82	90.57	2.60	87.60	2.50	84.39
SD			±0.03	±6.77	±0.03	±1.54	±0.06	±1.41

[a] 13.44 RU added per 908 g milk.
[b] Total and per cent activity of the theoretical activity in curd.

Table 10. Effect of pH of slurry on the recovery of EP enzyme[a] from curd formed at pH 5.2 (test performed on slurry-supernatant)

Replication	Activity recovered in whey	Theoretical activity in curd	Slurry supernatant					
			pH 5.4		pH 6.0		pH 6.8	
	(RU)	(RU)	(RU)	(%)	(RU)	(%)	(RU)	(%)
1	20.31	6.57	5.87	89.31	2.58	86.87	2.38	80.13
2	20.26	6.62	5.90	89.10	2.60	86.37	2.41	80.01
3	20.41	6.47	5.89	91.12	2.49	85.57	2.37	81.44
4	20.79	6.09	5.38	88.41	2.56	85.33	2.39	79.66
5	10.46	2.98	----	-----	2.58	86.58	2.44	81.87
6	10.56	2.88	----	-----	2.48	86.11	2.35	81.66
7	10.40	3.04	----	-----	2.60	85.52	2.43	79.93
8	10.45	2.99	----	-----	2.59	86.62	2.45	81.93
Mean	20.44	6.43	5.76	89.48	2.55	86.09	2.39	80.82
SD			±.23	±1.08	±0.43	±0.61	±0.15	±0.97

[a]13.44 RU added per 908 g milk. [b]Total and percent activity of the theoretical activity in curd.

Table 11. Effect of curd-water ratio of slurry at pH 5.4 on the per cent recovery of EP enzyme[a] from curd formed at pH 5.2 (test performed on slurry supernatant)

Replication	Activity recovered in whey	Theoretical activity in curd	Activity recovered in slurry supernatant[b]							
			1:5[c]		1:7[c]		1:11[c]		1:15[c]	
	(RU)	(RU)	(RU)	(%)	(RU)	(%)	(RU)	(%)	(RU)	(%)
1	20.41	6.47	5.90	91.19	5.83	90.10	5.85	90.41	5.81	89.79
2	20.27	6.61	5.95	90.01	6.03	91.23	5.93	89.71	5.93	89.71
3	20.49	6.39	5.63	88.10	5.61	87.79	5.55	86.85	5.51	86.22
4	20.71	6.17	5.66	91.73	5.41	87.68	5.51	89.30	5.53	89.62
Mean	20.47	6.43	5.78	90.26	5.72	89.20	5.71	89.06	5.70	88.83
SD			±0.03	±2.58	±0.07	±3.06	±0.05	±2.40	±0.04	±3.04

[a] 26.88 RU added per 1816 g milk.
[b] Total and per cent activity of the theoretical activity in curd.
[c] Curd-water ratio.

Table 12. Effect of NaCl in 1:5 curd-water slurries (pH 5.4) on the per cent recovery of EP enzyme[a] activity from curd formed at pH 5.2 (test performed on slurry)

Replication	Activity recovered in whey	Theoretical activity in curd	Activity recovered in slurry[b]					
			0.0% NaCl		0.5% NaCl		1.0% NaCl	
	(RU)	(RU)	(RU)	(%)	(RU)	(%)	(RU)	(%)
1	20.98	5.90	5.93	100.50	5.62	95.25	5.43	92.02
2	20.90	5.98	5.90	98.66	5.72	95.65	5.48	91.63
3	20.95	5.93	5.93	100.00	5.65	95.28	5.41	91.23
4	20.89	5.99	5.95	99.33	5.68	94.82	5.38	89.82
5	20.86	6.02	6.00	99.66	5.70	94.68	5.43	90.20
6	20.88	6.00	5.94	98.83	5.73	95.50	8.46	91.00
Mean	20.91	5.97	5.94	99.49	5.66	95.19	5.43	90.98
SD			±0.03	±0.70	±0.04	±0.38	±0.03	±0.84

[a] 26.88 RU added per 1816 g milk.
[b] Total and per cent activity of the theoretical activity in curd.

Table 13. Effect of NaCl in 1:5 curd-water slurries (pH 5.4) on the per cent recovery of EP enzyme[a] activity from curd formed at pH 5.2 (test performed on slurry supernatant)

Replication	Activity recovered in whey	Theoretical activity in curd	Activity recovered in slurry supernatant[b]					
			0.0% NaCl		0.5% NaCl		1.0% NaCl	
	(RU)	(RU)	(RU)	(%)	(RU)	(%)	(RU)	(%)
1	20.98	5.90	5.88	99.66	5.60	94.91	5.45	92.72
2	20.90	5.98	5.92	98.88	5.66	94.64	5.50	91.97
3	20.95	5.93	5.86	98.80	5.69	95.95	5.45	91.90
4	20.89	5.99	5.89	98.33	5.72	96.13	5.40	90.15
5	20.86	6.02	5.94	98.67	8.78	96.01	5.43	91.99
6	20.88	6.00	5.90	98.33	5.77	96.16	5.42	90.33
Mean	20.91	5.97	5.89	98.79	5.70	95.63	5.44	91.51
SD			±.03	±0.49	±0.07	±0.68	±.03	±1.02

[a] 26.88 RU added per 1816 g milk.

[b] Total and per cent activity of the theoretical activity in curd.

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