SURVIVAL OF PORCINE PEPSIN DURING CHEDDAR CHEESE MAKING AND ITS EFFECT ON CASEIN DURING CHEESE RIPENING

by

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A dissertation submitted in partial fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Nutrition and Food Sciences

Approved:

UTAH STATE UNIVERSITY
Logan, Utah
1984
ACKNOWLEDGEMENTS

I wish to extend appreciation and a very special thanks to Dr. C. A. Ernstrom for his great counsel, advice, encouragement and recommendations throughout this study.

Appreciation is expressed to Dr. R. J. Brown, Dr. Daren Cornforth, Dr. LeGrande Ellis and Dr. D. V. Sisson for their suggestions and considerations during this work. I am grateful to all the staff and graduate students at the department of Nutrition and Food Science for their help and kindness during my study.

Appreciation is extended to the University of Basrah-Iraq for their financial support throughout my whole study.

This study was supported with funds provided by the Dairy Research Advisory Board (DRAB) and administered through the Utah State University Experimental Station.

Finally, I am very indebted to my wife Anda and my two children Haydar and Ola for their patience, and to all my family members in Iraq for their encouragement.

Gheyath H. Majeed
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ABSTRACT

Survival of Porcine Pepsin During Cheddar Cheese Making and its Effect on Casein During Cheese Ripening

by

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Utah State University, 1984

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A modification of a linear diffusion test for measuring milk clotting enzymes at concentrations of $1 \times 10^{-4}$ to $1 \times 10^{-1}$ chymosin units/ml was developed to permit quantitative assay of porcine pepsin in Cheddar cheese with a standard deviation of 6%. The amount of porcine pepsin retained in Cheddar cheese curd was dependent on pH of milk at setting. Milk at pH 6.6, 6.4, 6.2, and 6.0 was set with porcine pepsin, acidified with lactic acid and glucono-δ-lactone and made into Cheddar cheese. After pressing the corresponding curd contained 0, 3.64±0.12%, 4.79±0.52%, and 5.94±0.30% of the pepsin activity added to the milk. Polyacrylamide gel electrophoresis of cheese revealed increasing degradation of the $\alpha_{s1}$-casein band with increasing residual pepsin in the curd. However, some degradation of the $\alpha_{s1}$-casein band was evident in curd set at pH 6.6 which showed no residual pepsin. Curd was made
by ultrafiltration from whole milk followed by acidification to pH 5.2 with hydrochloric acid and glucono-6-lactone and vacuum evaporated to 39% moisture at 40°C (no clotting enzymes and no starter). Degradation of the $\alpha_s$-casein in this curd was similar to that observed in pepsin cheese set at pH 6.6. All degradation in $\alpha_s$-casein cannot be attributed to milk clotting enzymes or starter bacteria. Porcine pepsin does not contribute to protein digestion in cheese curd during ripening unless the milk is below pH 6.6 at setting.
INTRODUCTION

Rennet (EC 3.4.23.4), the crude extract from the fourth stomach of young calves is the coagulant historically used in cheese making. During the last two decades a noticeable shortage of rennet has existed (28). This shortage is due to increased cheese production and decreased availability of young veal calves (80). This situation created an interest in finding other proteases that can substitute for rennet.

Porcine pepsin (EC 3.4.23.1) has been successfully used as a rennet substitute (14, 24, 33, 34, 42, 51, 53, 56, 57, 70, 73, 83). Its use has raised the question of the importance of milk clotting enzymes in cheese curing. Previous reports have suggested that porcine pepsin does not survive the Cheddar cheese making process (35, 39, 55), yet satisfactory cheese can be made with this enzyme (55, 83), which has very poor stability above pH 6.2 (23, 24). This argues that milk clotting enzymes play little if any significant role in Cheddar cheese curing. There was less proteolytic breakdown in Cheddar cheese made with porcine pepsin than that made with chymosin (EC 3.4.23.4) (the pure enzyme from rennet) (35, 53, 56, 83). This would be true if porcine pepsin was inactivated during the cheese making process. However, cheese quality was not significantly affected.
Porcine pepsin was rapidly denatured in phosphate buffer between pH 6.4 and 6.7 at a temperature between 31 and 39°C, but was more stable in milk under similar conditions (70). In Cheddar cheese making, porcine pepsin appeared to be more stable than in phosphate buffer or milk, but was affected by the pH at setting.

It has been claimed that porcine pepsin and chymosin are equally stable under normal cheese making conditions (70). This was based on observed proteolysis in cheese which was attributed to pepsin activity. However, degradation of $\alpha_{s1}$-casein during ripening of cheese set at pH 6.6 and 6.65 might have been due to the presence of a more stable chymosin-like enzyme in the pepsin preparation or to natural milk proteases. Other workers (35) concluded that porcine pepsin does not survive the Cheddar cheese making process.

A diffusion technique to measure residual milk clotting enzymes in cheese curd was developed by Holmes et al. (40). However, this technique failed to measure residual porcine pepsin in the curd because the pH of extraction of the enzyme must be at 6.8 which would inactivate porcine pepsin. This procedure can measure very low concentrations of milk clotting enzymes that are stable at pH 6.8 (down to $10^{-4}$ chymosin units/ml), and is considered very effective for measuring residual chymosin and micro-
bial proteases in cheese. Other procedures have been developed by other workers (19, 47, 55, 76, 78, 94) to measure low concentrations of milk clotting enzymes.

The purpose of this study was to:

1. Modify the Holmes et al. (40) procedure to enable complete recovery of pepsin from curd without loss of activity.
2. Determine whether porcine pepsin survives the Cheddar cheese making process.
3. If pepsin does not survive the cheese making process, explain the cause of $\alpha_{s1}$-casein decomposition in cheese reported by O'Keeffe et al. (70).
4. If porcine pepsin does survive the cheese making process, determine its effect on caseins during cheese ripening.
Cheese ripening

Ripening of cheese usually refers to the biochemical and physical changes in cheese to give the desired flavor and texture. These changes are achieved by a very complicated series of biological and biochemical reactions which involve a number of enzymatic reactions. Their extent depends on many factors such as moisture content of cheese, temperature of ripening, and pH of the cheese. Although many studies and reviews concerning this subject have been published (1, 37, 54, 81), many aspects of cheese ripening have not been explained.

Cheese ripening includes changes in the main components of cheese, which are, carbohydrates, lipids, and casein. In this review section, emphasis will be on casein changes caused by residual milk clotting enzymes retained in the curd. Effects of starter proteinases have been reviewed extensively by other workers (37, 54, 81).

Carbohydrate changes are insignificant during cheese ripening. The reactions involving lactose are mostly during the cheese making process and during the early period of ripening to form lactic acid. However, trace amounts of lactose remain in cheese curd which is converted to lactic acid by the glycolysis pathway (37). Adda et al. (1) mentioned that carbohydrates are fermented through the...
Hexose diphosphate pathway to pyruvic acid and then to lactic acid. Conversion of lactose to lactic acid is essential to normal cheese flavor and texture. Formation of lactic acid inhibits the growth of undesirable microorganisms (81). The acidity of the curd causes solubilization of phosphate and calcium. Calcium level is an important factor in cheese texture and body (1).

Cheese fat contribution to Cheddar cheese flavor has been studied by Ohern and Tuckey (69). They found that Cheddar cheese made from skim milk did not have typical Cheddar cheese flavor. Typical Cheddar cheese flavor required a balance of free fatty acids and acetate. The best ratio of free fatty acids to acetate was 0.55 to 1.0.

Degradation of casein during cheese ripening

Caseinate undergoes a series of changes during ripening of cheese to give the required body, texture and flavor of cheese. These changes are due to proteolytic enzymes present in cheese. Sources of these enzymes are: milk clotting enzymes, proteolytic enzymes from starter bacteria, proteases from contaminating bacteria, and natural milk proteases.

Role of milk clotting enzymes

Milk clotting enzymes used in cheese making contribute to proteolytic breakdown during cheese curing (10, 14, 30,
34, 35, 48, 65, 70, 71, 89, 90, 92). However, the evidence is inconclusive as to whether this enzymatic effect is contributory, detrimental, or insignificant to overall cheese quality and flavor development. Much research has been done to evaluate the role of milk clotting enzymes in cheese curing (10, 11, 14, 15, 25, 32, 35, 49, 56, 66, 70, 71, 72, 82, 83, 89, 90, 91, 92). The action of milk clotting enzymes on casein has been reported by many authors (2, 6, 29, 30, 50, 60, 62, 63, 93).

Visser (90) studied protein breakdown in asceptically made Gouda cheese. Action of chymosin and starter bacteria were investigated during cheese ripening. Chymosin was responsible for most of the soluble nitrogen produced. Starter bacteria proteases and milk protease in the coagulant-free cheeses had less effect. Chymosin contributed to the production of high and low molecular weight peptides, while starter bacteria proteases contributed to the production of low molecular weight peptides (<1400) independent of chymosin and dependent on the kind of culture used. Milk protease produced low molecular weight peptides and amino acids independent of chymosin or starter bacteria. He concluded that when all enzymes were acting together in normal cheese, high molecular weight peptides produced by chymosin were degraded by starter proteases to form low molecular weight peptides and amino acids. These
results agree with those of other workers (48, 71).

A gel electrophoretic study was done by Visser et al. (92) under the above conditions, which showed that $\alpha_{s1}$-casein degradation occurred rapidly in normal asceptic Gouda cheese, while $\beta$-casein degradation was slow. It appeared from the starter free cheese that chymosin was responsible for degrading $\alpha_{s1}$-casein and $\beta$-casein during the first month of ripening. Starter bacteria proteases degrade $\alpha_{s1}$-casein and $\beta$-casein significantly but slowly. Milk protease was responsible for the formation of "minor caseins" from $\beta$-casein and also degraded $\alpha_{s1}$-casein, which appeared clearly on the electrophoretic patterns of starter-free and rennet-free asceptic cheese after 6 months of ripening. However, chymosin was the determinative agent for the extent of proteolysis in Gouda cheese.

Ripening of Noodhollandse Meshanger cheese (soft cheese) was studied in detail by Noomen (65,66). He concluded that presence of surface flora is not necessary for the softening of this type of cheese. Results with glucono-δ-lactone acidified cheese showed that even though lactic acid bacteria contribute to protein degradation during ripening, they are still not crucial for the changes in consistency in the cheese. The proteolytic activity of chymosin was the main cause of protein degradation in Meshanger cheese softening.
Chymosin degraded $\alpha_{s1}$-casein much more than $\beta$-casein in soft type cheese. The action on $\alpha_{s1}$-casein was pH dependent with maximum activity near pH 5.0 (66). However, sodium chloride concentration also affected the degradation of $\alpha_{s1}$-casein. Up to 4% of sodium chloride in cheese moisture stimulated the degradation of $\alpha_{s1}$-casein, but greater concentrations had a contrary effect. The effect of sodium chloride concentration on the degradation of $\alpha_{s1}$-casein and $\beta$-casein by chymosin and porcine pepsin was studied in 3% casein in 0.1 M phosphate buffer at pH's 6.5, 6.0, and 5.2 (30). Optimum degradation of $\alpha_{s1}$-casein by chymosin occurred in the presence of 5% sodium chloride, and by porcine pepsin at 5-10%. However, 50% of $\alpha_{s1}$-casein was hydrolyzed by chymosin in the presence of 20% sodium chloride. Proteolysis of $\beta$-casein by both enzymes was inhibited by 10% sodium chloride, and was reduced in the presence of 5% sodium chloride. The inhibitory effect of sodium chloride on the proteolysis of casein was independent of pH and incubation temperature.

Activity of milk protease in soft cheese (Meshanger type) appeared to be affected by pH and sodium chloride concentration (65). At low acidity (pH 6.2) protein degradation by milk protease was more extensive than at high acidity (pH 5.4). Degradation of $\alpha_{s1}$-casein by milk protease at pH 6.2 was slower than that of $\beta$-casein. At pH
5.4, $\alpha_{\text{s1}}$-casein was degraded more than $\beta$-casein by milk proteases. The higher concentration of sodium chloride in cheese decreased the degradation of both caseins. However, the contribution of milk proteases to protein degradation in Meshanger cheese was of little importance for the normal cheese ripening. The relation between the breakdown in $\alpha_{\text{s1}}$-casein and consistency of Meshanger cheese has been discussed by de Jong (15). The best consistency was when 70% of the $\alpha_{\text{s1}}$-casein was degraded.

The specificity of chymosin to $\alpha_{\text{s1}}$-casein was studied by several workers (12, 13, 62, 63). Chymosin hydrolyzed $\alpha_{\text{s1}}$-casein to $\alpha_{\text{s1}}$-I, $\alpha_{\text{s1}}$-II, and $\alpha_{\text{s1}}$-III/IV in diluted phosphate buffer at pH 5.8 or less (63). At pH 4.6 however, chymosin hydrolyzed $\alpha_{\text{s1}}$-casein to $\alpha_{\text{s1}}$-I, then $\alpha_{\text{s1}}$-V. Proteolytic specificity was altered by sodium chloride. At 5% w/v sodium chloride solution and pH 5.2, $\alpha_{\text{s1}}$-casein was hydrolyzed to $\alpha_{\text{s1}}$-I, then $\alpha_{\text{s1}}$-VII and $\alpha_{\text{s1}}$-VIII.

The bonds most labile to chymosin in $\alpha_{\text{s1}}$-casein in Cheddar cheese were bond 23-24 and 24-25 (13). The $\alpha_{\text{s1}}$-I casein consists of residues 25-199 of $\alpha_{\text{s1}}$-casein. The specificity of chymosin to $\alpha_{\text{s1}}$-casein was dependent on the pH of reaction and the state of aggregation (63). The rheological properties of young Cheddar cheese was linked to the conversion of $\alpha_{\text{s1}}$-casein to $\alpha_{\text{s1}}$-I casein (12).
It has been suggested that proteases from *Mucor miehei* and *Mucor pusillus* var. Lindt are used extensively in cheese making only because such a small amount remains in the curd (2-3%) (40). *Endothia parasitica* is used for Swiss cheese, but no activity has been reported in the cheese where high cooking temperature was employed (79). These fungal enzymes have greater proteolytic activity than rennet or pepsin as measured by soluble nitrogen when using fresh cheese as a substrate or when incubated in casein solution (60). The electrophoretic patterns also showed differences in proteolytic action on cheese between pepsin, fungal proteases and chymosin (60). Bendet and Park (2) found that *Endothia parasitica* enzyme degraded $\alpha_s$-casein more than *Mucor miehei* enzyme or chymosin as determined by sodium dodecyl sulfate poly acrylamide gel electrophoresis on acid casein. However, the effect of the three enzymes on $\beta$-casein was insignificant. $\kappa$-casein degradation by the three enzymes was similar.

Action of milk clotting enzymes on $\beta$-casein has been reported by many authors (6, 20, 49, 50, 95). Cerbulis et al. (6) found that both chymosin and porcine pepsin degraded $\beta$-casein more than chymosin when given sufficient time. Mickelsen and Fish (60) found that degradation of $\beta$-casein was caused by chymosin, porcine pepsin and fungal rennet when added to whole casein solu-
tion. However, Ledford et al. (49) did not notice any effect of chymosin on $\beta$-casein of Cheddar cheese after 15 days ripening, as shown by their electrophoretic patterns of Cheddar cheese made without starter. Degradation of $\alpha_{s1}$-casein occurred after overnight pressing. However, the physical form of casein influenced rate of proteolysis by chymosin (50). Degradation of dissolved casein was faster than of casein micelles when milk was the substrate. The dissolved casein showed a quick proteolysis of $\alpha_{s1}$-casein and $\beta$-casein which agrees with Cerbulis et al (6). In milk $\alpha_{s1}$-casein was also degraded very rapidly while $\beta$-casein seemed to resist proteolysis.

The action of chymosin on $\beta$-casein has been studied extensively by Visser and Slangen (93). $\beta$-casein was attacked by chymosin at positions (in order of decreasing susceptibility): Leu (192)-Tyr (193) > Ala (189)-Phe (190) > Lue (165)-Ser (166) > Lue (127)-Thr (128). This was studied at 13 C in 0.05 M sodium acetate buffer at pH 5.4.

Degradation of $\beta$-casein by chymosin in Cheddar cheese and Gouda cheese was studied by Creamer (10). He attributed the formation of $\lambda$-caseins to the action of starter enzymes or and milk protease. The degradation of $\beta$-casein was more extensive in Gouda cheese than in Cheddar cheese, while the degradation of $\alpha_{s1}$-casein was more extensive in Cheddar cheese than in Gouda cheese.
This was attributed to the pH of cheese since Cheddar has a lower pH than Gouda cheese, which is more optimum to chymosin action on $\alpha_{s1}$-casein.

Formation of bitter peptides in cheese during ripening has been investigated by many workers (48, 71, 89, 91). Lawrence et al. (48) suggested that chymosin produced high molecular weight non-bitter peptides during Cheddar cheese ripening, which were consequently altered by starter bacteria proteinases to small molecular weight bitter peptides. O'Keeffe et al. (71) concluded that chymosin and pepsin were responsible for formation of large peptides during Cheddar cheese ripening, while starter bacteria proteinases were responsible for formation of small peptides and amino acids from larger peptides produced by the milk coagulant. Visser (89, 91) also claimed that bitterness in Gouda cheese might be caused by small molecular weight peptides (<1400). He concluded that chymosin was capable of producing bitter peptides in Gouda cheese after some months of ripening. Milk protease did not contribute to bitterness in Gouda cheese.

Acid proteases of bovine milk which have been purified by Kaminogawa et al. (43) degraded $\alpha_{s1}$-casein, $\beta$-casein, and $\kappa$-casein (44) to a fragment that had the same mobility in disk and urea sodium dodecyl sulfate electrophoresis as that caused by the action of chymosin. However, para
\( \kappa \)-casein-like proteins that were formed by the action of acid proteases formed much slower than para-\( \kappa \)-casein formed by chymosin.

**Effect of porcine pepsin during cheese ripening**

The effect of porcine pepsin on caseins during ripening of cheese has been studied by many workers (14, 29, 56, 70, 83).

Davies et al. (14) found that variations in the amount of pepsin had an insignificant effect on flavor, texture, body or ripening of Cheddar cheese. It was concluded that pepsin produced less protein degradation in Cheddar cheese than chymosin, however, pepsin produced good quality Cheddar cheese (51, 56, 57, 70, 83). Melachouris and Tuckey (56) investigated the differences in proteolysis produced by commercial rennet extract (chymosin) and porcine pepsin preparation (Metroclot) during ripening of Cheddar cheese. The proteolysis produced by chymosin was higher than that produced by porcine pepsin as was evident by non-protein nitrogen. However, both enzymes produced good quality Cheddar cheese.

Milk clotting activity of porcine pepsin is highly dependent on pH of milk at setting (23, 95). Ernstrom (23) found that milk did not coagulate with porcine pepsin at pH 6.8 even though it was allowed to act for over two h.
Fox (29) studied milk clotting and proteolytic activity of chymosin, bovine pepsin, and porcine pepsin. Coagulation did not occur above pH 6.68 when porcine pepsin was the milk coagulant. Bovine pepsin was slightly more dependent on pH than chymosin. However, it was more stable to high pH than porcine pepsin of equal milk clotting activity. Porcine pepsin had greater proteolytic activity than chymosin or bovine pepsin, in sodium caseinate buffered in 0.1 M sodium phosphate as a substrate. Green (33) found that the ratios of milk clotting activity to proteolytic activity were high for chymosin and bovine pepsin but low for porcine pepsin. However in Cheddar cheese ripening, cheese proteolysis caused by porcine pepsin was less than that of chymosin, which they attributed to loss of porcine pepsin activity during Cheddar cheese making. They concluded that porcine pepsin was suitable for Cheddar cheese making if ripening time is increased.

A study of the survival of porcine pepsin during Cheddar cheese making was conducted by O'Keeffe et al. (70). Porcine pepsin was denatured easily in phosphate buffer at pH 6.4 to 6.7 between 31 and 39 C. However, it was more stable in milk under similar conditions. Porcine pepsin was more stable in Cheddar cheese making than in phosphate buffer or milk and its activity was affected by
the pH of milk at setting. They claimed that porcine pepsin was equally as stable as chymosin under normal cheese making conditions, and contributed as much as chymosin to Cheddar cheese proteolysis during ripening. Stability of porcine pepsin during Cheddar cheese making was attributed to:

1. Failure of the interior curd particles to attain cooking temperature (39°C) as quickly as expected.
2. Adsorption of the enzyme to casein made it less susceptible to denaturation.

However, they did not mention the effect of bacterial contamination during cheese making that may have caused degradation of $\alpha_s1$-casein during ripening of starter free cheeses. Moreover, degradation of $\alpha_s1$-casein might have been caused by more stable chymosin like enzymes in the pepsin preparation, or by natural milk proteases.

Cheddar cheese milk was acidified with lactic acid and glucono-6-lactone (no starter) and was set with porcine pepsin or chymosin at pH's 6.65, 6.6, 6.55, 6.5, and 6.4. The lower the pH at setting, the more degradation occurred during ripening of Cheddar cheese as shown by their polyacrylamide-urea gel electrophoretic patterns. For those cheeses set at pH 6.4, 6.5, and 6.55, degradation in $\alpha_s1$-casein appeared after pressing, whereas for those cheeses set at pH's 6.65, 6.6, degradation in $\alpha_s1$-casein...
was evident after 1 week and 6 weeks ripening (Figure 1). Their results, however, contradicted other workers (35, 40, 56) who claimed that porcine pepsin might be destroyed either partially or completely during cheese making. However, the evidence as to whether porcine pepsin survives the Cheddar cheese making process and its role in cheese ripening was not explained or clarified in the previous studies.

**Porcine pepsin as a rennet substitute**

The idea of using a substitute for rennet in the cheese industry was raised when a shortage in rennet existed. Any rennet substitute should be safe for human consumption and relatively inexpensive. Porcine pepsin is one of the proteases that has been successfully used as a rennet substitute (14, 42, 56, 70), but the Research Committee of the National Cheese Institute recommended that porcine pepsin not to be used as a complete rennet substitute (64). Many reviews about the milk coagulants have been published (24, 34, 80).

A significant problem of porcine pepsin is the slow coagulation at high pH values (>6.6) (23, 24, 95) when used in cheese making. However, pepsin may be used in combination with rennet in cheese making (7, 73, 79).

Porcine pepsin belongs to the group of aspartic pro-
Figure 1. Electrophoretograms of chemically acidified cheeses set at pH 6.4, 6.5, 6.55, 6.6, or 6.65 after pressing (slots 1, 2, 3, 4 and 5), after 1 week ripening at 7°C (slots 6, 7, 8, 9 and 10 respectively) and after 6 weeks ripening at 7°C (slots 11, 12, 13, 14 and 15 respectively) (70).
teases (EC 3.4.23) (45), which has a homologous structure. (27). It consists of two domains with the active center in the cleft between the domains. The complete sequence of porcine pepsin has been published (27). The amino acid sequence shows about 50% difference relative to chymosin. Porcine pepsin has been purified and crystallized by many workers (38, 67, 68, 78).

**Porcine pepsin stability**

Milk clotting activity of porcine pepsin was studied by Ernstrom (23). Porcine pepsin activity declined as the pH of milk increased. No coagulation of skim milk occurred when the setting pH was 6.8. Mickelsen and Ernstrom (59) showed the effect of pH on the stability of chymosin-porcine pepsin blends. At pH 7.3 and 30 C, porcine pepsin was deactivated rapidly and completely, while chymosin was affected slightly. However, at pH 5.5 pepsin and chymosin had the same stability. The greatest stability of porcine pepsin in chymosin-pepsin blend was 3.8 and 5.5. Loss of pepsin activity occurred at pH 6.6 and higher. Although chymosin alone was not stable at pH 3, with 25% or more pepsin, chymosin was destroyed completely in 48 h by porcine pepsin at pH 3. At pH 6.5, milk clotting activity of 100% porcine pepsin was completely destroyed after 48 h at 30 C. Factors affected stability of chymosin also were investigated (58).
The effect of temperature on porcine pepsin with relation to pH has been studied by Edelhoch (17) and O'Keeffe et al. (70). Porcine pepsin was denatured rapidly in phosphate buffer at pH 6.4 to 6.7 between 31 and 39°C (70). Edelhoch found that in KNO₃ neutral salt solution, the heat of inactivation of porcine pepsin declined uniformly between pH 6 and 6.7. Inactivation of porcine pepsin in hard water was studied by Emmons (20). Porcine pepsin was denatured quickly at pH higher than 6.5 when diluted with hard water at ambient temperature. It was concluded that a temperature of less than 20°C and pH of less than 6.5 should be used to maintain activity of porcine pepsin during dilution with hard water. However, porcine pepsin was very stable in 5M urea at pH 2 and 2.5 at 30°C, but chymosin, Mucor miehei protease, and Mucor pusillus var. Lindt protease were denatured rapidly under the same conditions (61).

Porcine pepsin stability versus chymosin and other pepsins from rabbit and sheep was studied by El-Abbassy and Wahba (18). Porcine pepsin was mostly affected by pH changes. However, addition of CaCl₂ increased the clotting activity of all enzymes, whereas sodium chloride decreased clotting activity.
Microtests of milk clotting enzymes

Many methods have been developed to measure milk clotting activity of milk coagulants (8, 9, 16, 21, 22, 47, 52, 74). However, the method reported by Ernstrom (21, 22) remains the preferred assay method by the rennet industry. This method used the Sommer-Matsen apparatus (84), which has been developed to test the clotting time of mastitic milk, and Berridge substrate (12 g non-fat dry milk in 100 ml 0.01 M CaCl$_2$) (3). The activity in the unknown solution was calculated by the following equation:

$$\frac{Ts}{Cu} = \frac{Cs}{100} = \frac{Cu}{Tu}$$

where:

- $CU/ml =$ Chymosin units/ml of unknown
- 100 = Chymosin units in 1 ml of diluted standards
- $Ts =$ Clotting time of standard
- $Tu =$ Clotting time of unknown
- $Cs =$ Concentration of standard
- $Cu =$ Concentration of unknown

McMahon and Brown (52) evaluated the Formagraph for measuring milk clotting activity of chymosin in comparison to a standard. A diagram of firmness versus time was recorded on photographic paper. This method does not require continual observation. However, coagulation time
by this method is longer than that measured by the conventional method (22). Both methods give linear standard curves between 0.2 and 1 chymosin units/ml enzyme concentration. Milk-clotting enzyme activity can be measured equally well by both methods.

Cheeseman (8) developed a technique to measure milk clotting activity of milk proteases by their diffusion in an agar gel containing 1% whole casein and 10-20 mM CaCl₂. However, the sensitivity of this technique is less than that of Berridge substrate (3). The Cheeseman technique was applied to study the action of milk clotting enzymes prepared from cow, buffalo, vegetable, and microbial sources on caseins from cow and buffalo milk (31). Milk coagulants from cow and buffalo formed two precipitating zones while diffusing through an agar plate containing the Cheeseman diffusion substrate. However, milk coagulants prepared from vegetables and microorganisms exhibited different action on a casein agar plate forming only one precipitating zone followed by a clear zone. The differentiation of enzyme coagulants on casein-agar gels of pH 6.85 was studied by Richardson (77). Chymosin produced two precipitating zones after 24 h incubation at 32 C. Porcine pepsin produced a primary zone with smaller diameter. However, coagulants from Endothia parasitica and Mucor pusillus var Lindt produced single zones. It was concluded
that casein-agar gel techniques cannot replace the standard milk clotting tests for measuring coagulants activity.

Lawrence and Sanderson (47) modified the Cheeseman technique to measure the activity of low concentrations of milk coagulants. An agar diffusion assay using calcium caseinate was very sensitive and suitable for qualitative and quantitative assay for chymosin and other milk coagulants. However, this test did not permit measurement of chymosin in all cheese and whey samples (39). Moreover, the procedure required humidity control to prevent the agar gel from drying during incubation.

Holmes (39) developed a technique for quantitating low concentrations of clotting enzymes in a substrate similar to that described by Lawrence and Sanderson (47), which was based on the gel diffusion technique developed by Caseman and Bennet (5), and Hall et al. (36) to detect and quantitate enterotoxins in foods. The Lawrence and Sanderson substrate was modified to increase its sensitivity. The modified substrate contained 0.5% casein, 3.6% sodium acetate, 0.01% CaCl$_2$, and 0.7% Ion agar. Sedimentation tubes (3 mm ID X 110 mm length) were 3/4 filled with the substrate. Five microliters of the enzyme solution was applied to the top of the diffusion tubes (after they were solidified by refrigeration, and brought
back to room temperature). The inoculated tubes were sealed and incubated at 37 C for 48 h. The precipitating bands formed in the casein-agar gel were measured by a densitometer. Concentrations of the unknown were determined by a standard curve prepared from known enzyme concentrations on semilogarithmic graph paper (Figure 2).

Holmes diffusion technique can measure concentrations as low as 1x10^{-4} chymosin units/ml with a 5% standard deviation. However, at higher concentrations, the conventional clotting test with Berridge substrate is more precise and convenient.

The diffusion technique was later improved by Holmes et al. (40). Whole casein was replaced with β-casein and calcium salts were eliminated to prevent cloudiness of the diffusion gel during storage.

The distribution of chymosin, porcine pepsin, Mucor pusillus var Lindt protease, Mucor miehei, and chymosin pepsin mixture between curd and whey was determined in milk set at pH 5.2, 6.0, 6.4, and 6.6, by the diffusion technique (40). The distribution of chymosin and porcine pepsin between whey and curd was pH dependent. The lower the pH at setting the more chymosin or porcine pepsin activity would be retained in the curd. There was a retention of 31% and 86% in the curd of freshly coagulated milk of the total chymosin activity added to milk when the pH
Figure 2. Standard curve (concentration vs 48 h diffusion distance) of chymosin and Mucor pusillus protease (40).

Figure 3. Distribution of chymosin between curd and whey from freshly coagulated milk (40).

Figure 4. Distribution of Mucor pusillus protease between curd and whey from freshly coagulated milk (40).
of setting was 6.6 and 5.2 respectively (Figure 3). The amount of chymosin retained in whey, was 72% and 17% respectively. However, the distribution of *Mucor pusillus* var Lindt protease and *Mucor miehei* protease was independent of pH at setting. About 17% and 83% of total enzyme activity added to milk was retained in curd and whey at all pH values (Figures 4 & 5). Porcine pepsin distribution between curd and whey was affected by pH of milk at setting in a manner similar to chymosin (Figure 6). However at pH values greater than 6.5 pepsin is unstable, therefore much of the activity was lost. Also, in order to release pepsin from curd, the curd slurry had to be adjusted to pH 6.8 which inactivated the enzyme.

The survival of milk clotting enzymes during Cheddar cheese making was also reported (39, 40). It was found that 7% and 58% of the original chymosin added to cheese milk (Figure 7), 6% and 93% of the original *Mucor pusillus* protease added to cheese milk (Figure 9), and 5% and 17% of the original chymosin-pepsin mix (Figure 8) was active in the curd and whey respectively at dipping. After overnight pressing, 6% of the chymosin, 3% of *Mucor pusillus* protease, 1.8% of *Mucor miehei* protease (Figure 10), and 4% of the chymosin-pepsin mix was retained in cheese curd. However, only 9% of the total porcine pepsin added to cheese milk was detected in the whey at dipping, and no
Figure 5. Distribution of Mucor miehei protease between curd and whey from freshly coagulated milk (40).

Figure 6. Distribution of porcine pepsin between curd and whey from freshly coagulated milk (40).

Figure 7. Distribution of chymosin between curd and whey during Cheddar cheese making (40).

Figure 8. Distribution of commercial rennet-pepsin mix between curd and whey during Cheddar cheese making (40).
Figure 9. Distribution of Mucor pusillus protease between curd and whey during Cheddar cheese making (40).

Figure 10. Distribution of Mucor miehei protease between curd and whey during Cheddar cheese making (40).

Figure 11. Distribution of porcine pepsin between curd and whey during Cheddar cheese making (40).
activity was detected in cheese curd after pressing (Figure 11).

Stadhouders et al. (85) developed an extraction procedure to quantitate the amount of chymosin retained in cheese curd. Large amounts of 1M acetate buffer at pH 4.9 were used to extract the enzyme from cheese curd at 25 C for 16 h. The extract was then centrifuged and dialized against distilled water, then freeze dried. The extract powder was dissolved in water at 30 C and mixed with an equal volume of double reconstituted milk prepared from low heat skim milk powder to estimate the clotting time, against a standard with a known amount of chymosin. However, this procedure has not been applied to other milk clotting enzymes including porcine pepsin. The method seems difficult and complicated in comparison to that of Holmes et al. (40). Moreover, it might be inconvenient to apply to porcine pepsin because the time of extraction is too long (16 h at 25 C), which might inactivate some of the enzyme.

A sensitive immunochemical assay method to determine the amount of chymosin in cheese has been developed by Matheson (55). It is a combination of immunochemical and diffusion methods. Chymosin can be determined by this procedure qualitatively and quantitatively even though
other milk coagulant enzymes of similar specificity are present with the chymosin in cheese.

It appeared that Holmes et al. (40) procedure for measuring low concentrations of milk clotting enzymes is the most convenient method to measure the residual porcine pepsin quantitatively in Cheddar cheese curd. However, a modification of the extraction procedure is required to enable a complete recovery of the enzyme (porcine pepsin) from the curd.

**Pepsin from other sources**

Bovine pepsin and chicken pepsin have been used as rennet substitutes in cheese making (29, 32, 33, 41, 87). Clotting activity of bovine pepsin was slightly more dependent on pH than that of chymosin (29). The proteolytic activities of bovine pepsin and chymosin were about the same.

Chicken pepsin was used unsuccessfully in Cheddar cheese making (33, 87). Purification and characterization of chicken pepsin was described by Bohak (4). Cheddar cheese made with chicken pepsin was unacceptable in quality and yield (33, 87). Chicken pepsin was more stable than calf chymosin or porcine pepsin and has about the same stability as that of Mucor miehei protease at pH 6.3 and 50 °C (33). The efficacy of chicken pepsin as a milk clotting enzyme was studied by Gordin and Rosenthal
(32). They found that good quality Emmenthal, Kashkaval and unripened soft cheeses were produced with chicken pepsin when attention was paid to pH control and temperature, and with slight modification in the cheese making technique. Husek and Dedek (41) claimed that, during cheese making, with partial substitution of chymosin by chicken pepsin, the ripening was faster. Partial substitution of chymosin with chicken pepsin up to 30% was adequate to produce good quality Edam cheese, Moravian Loaf cheese (Emmenthal type cheese), and Zlato (a Bel Paese type of cheese).
MATERIALS AND METHODS

Milk

Raw whole milk was obtained from Utah State University Dairy Laboratory and pasteurized at 63°C for 30 min, adjusted to the desired pH by lactic acid or concentrated hydrochloric acid, and kept overnight at 2°C.

Enzymes

Standard rennet (100 chymosin units/ml) was obtained from New Zealand Cooperative Rennet Co., Ltd. Porcine pepsin was obtained as a crude powder (activity 1200-2000 units/mg protein. One unit will produce a $A_{280}$ of 0.001 per min at pH 2.0, measured as TCA-soluble products using hemoglobin as a substrate), and crystalline powder (activity 2500-3200 units/mg protein) from Sigma Chemical Company. The activity of 3.75% of crude powder pepsin solution was evaluated against the standard rennet at pH 6.2 and 6.5 by the method of Ernstrom (22). The activity of 0.5% of crystalline porcine pepsin solution was evaluated against standard rennet at pH 6.5.

Glucono-δ-lactone

Glucono-δ-lactone was obtained as a coarse powder from Pfizer Chemical Division (Pfizer Inc. New York, N.Y.)

Starter

Starter was obtained from Biolac Inc. (Logan, Utah).
Diffusion tubes and test substrate

The diffusion tubes filled with the test substrate were obtained from Biolac Inc. (Logan, Utah), or prepared according to Holmes et al. (40).

Measurement of enzyme concentrations

Whey:

Measurement of the enzyme concentrations in whey was according to Holmes et al. (40), except that, the diffusion distance was measured with a Beckman DU-8 spectrophotometer. A standard curve was prepared for chymosin and porcine pepsin as in Holmes et al. (40). Diffusion distances of known enzyme concentrations ($10^{-1}$, $10^{-2}$, $10^{-3}$, and $10^{-4}$) were plotted versus enzyme concentrations on semi-logarithmic graph paper. Concentration of the unknown can be determined from this curve.

Curd:

Measurement of enzyme concentration in curd was according to Holmes et al (40) for the control. The following modification was made on the extraction procedure of Holmes et al. (40) diffusion technique to allow complete recovery of porcine pepsin from curd.

Modification of Holmes et al. (40) diffusion technique

Thirty grams of curd were blended with 450 ml 1M NaCl
solution at slow speed in a Waring blender for 1.5 min to form the curd extract. The pH of the curd extract of freshly coagulated milk was adjusted to pH 6.0 with 1M HCl. The pH of the curd extract of Cheddar cheese was adjusted to pH 6.0 with 0.1M NaOH, then filtered by gravity using Whatman NO. 1 filter paper. For porcine pepsin, the extraction was done at <10°C to avoid any denaturation of the enzyme that may occur at higher temperatures (20).

Five microliters of the filtrate was applied to the top of the diffusion tube, then incubated for 48 h at 37°C (Figure 12). Enzyme concentration was determined from the standard curve.

**Measurement of diffusion distance**

The diffusion distance of chymosin and porcine pepsin was measured after incubation of the diffusion tubes at 37°C for 48 h by gel scanning using a Beckman DU-8 Spectrophotometer (Beckman Instruments, Inc., Irvine, California) (Figure 13). The diffusion distance of chymosin and porcine pepsin is the distance from the origin (meniscus of the κ-casein agar gel) to the leading edge of the precipitation band (Figure 14).

**Cheddar cheese**

Normal Cheddar cheese (control) was made according to Holmes (39). Starter-free Cheddar cheese was made as in
Figure 12. A schematic of the Holmes et al. (40) diffusion technique and the modified diffusion technique.
Raw whole milk

Pasteurized at 63 C for 30 min.

454 g

ORIGINAL PROCEDURE (Control)

225 g

Incubated for 15 min at 30 C after the first sign of coagulation.

Centerfuged at 3500 g for 20 min

wt. and vol. of curd was measured by difference.

Blended at low speed in Waring blender for 1.5 min.

adjusted to pH 6.8 and left for 30 min at room temp.

Filteration

diff. tube

(Incubated for 48 h at 37 C)

MODIFIED PROCEDURE

225 g

Curd

Whey

5 μl

30 g curd + 450 ml water

curd slurry

adjusted to pH 6.0

Filteration

diff. tube

30 g curd + 450 ml 1M NaCl solution

adjusted to pH 6.0

Filteration

diff. tube

diff. tube

diff. tube
Figure 13. Beckman DU-8 spectrophotometer used to measure enzyme diffusion distance by gel scanning.
Figure 14. Spectrophotometer tracing of density changes in a diffusion tube after 48 h incubation at 37 C with 5 µl of .1 CU/ml of chymosin.
O'Keeffe et al (72) with some modifications:

1. Cheddar cheese was made in 20 cm. cubical plastic vats (39). Milk was acidified by lactic acid to the desired pH (pH 6.6, 6.4, 6.2, and 6.0).

2. Heating was accomplished by electrical resistance (39).

3. Enzymes were added to milk at a rate of 67.5 ml of 100 chymosin units/ml per 454 kg milk. The amount of 3.75% porcine pepsin solution added to milk of pH 6.6 and 6.4 was based on evaluation against standard rennet (100 chymosin units/ml) at pH 6.5. The amount of 3.75% porcine pepsin solution added to milk of pH 6.2, and 6.0 was based on evaluation of 3.75% porcine pepsin solution against standard rennet at pH 6.2. The setting temperature was 30 C. The setting time was 30 min.

4. Curd was cut into cubes of about 1 cm³ in size.

5. Cooking: after cutting, the curd was held for 15 min with gentle agitation, then the temperature was raised from 30 to 38 C over a 30 min period. The curd was held at 38 C for 1 h with stirring. The pH of the curd and whey was adjusted with lactic acid each 10 min during cooking to that pH of normal Cheddar cheese (control). The amount of lactic acid added depended on the pH at setting.

6. Cheddaring, milling and pressing: After drawing the whey, 18 g of solid glucono-δ-lactone/450 g starter-free curd was added. Curd was made into one pile and was cheddared until the pH of the curd reached about 5.3.
Additional glucono-δ-lactone was added to cheese curd depending on the pH of the curd. Approximately, 3 g glucono-δ-lactone/450 g curd was required to decrease the pH by 0.1 pH unit (72).

7. Pressing was accomplished in small aluminium hoops with weights placed on top.

8. Ripening was accomplished at 7 C.

**Measurement of residual pepsin and chymosin in cheese curd**

Residual porcine pepsin and chymosin were measured in Cheddar cheese after pressing by the modified Holmes et al. diffusion technique (40).

**Ultrafiltered (UF) cheese curd**

Whole milk was pasteurized at 63 C for 30 min, then adjusted to pH 5.8 by concentrated hydrochloric acid, kept over night at 2 C, then adjusted again to pH 5.8 before ultrafiltration. Ultrafiltration was according to Ernstrom et al. (26), using Crepaco Food Equipment and Refrigeration ultrafiltration unit (Crepaco Inc. Chicago Ill.) (Figure 15). The retentate was pasteurized at 63 C for 30 min, then one liter was used first to measure the amount of glucono-δ-lactone required to reduce the pH to 5.1-5.2. The whole retentate was then adjusted to pH 5.2 by means of glucono-δ-lactone. Condensation of the retentate was by vacuum evaporation at 40.5 C until moisture
Figure 15. Crepaco ultrafiltration unit used for ultrafiltration and difiltration of whole milk (5X).
reached 39.3% (75) using Groen condenser (Groen, Inc. U.S.A) (Figure 16). A schematic of UF-cheese curd making is shown in Figure 17.

**Total bacterial count**

Total bacterial count of UF cheese curd was determined after 6 weeks and 12 weeks ripening to evaluate the effect of contaminating bacteria on casein degradation during ripening (86).

**Gel electrophoresis**

Cheese acidified with glucono-δ-lactone was examined for casein degradation during ripening by means of polyacrylamide urea gel electrophoresis at pH 9.1-9.3, using the procedure of Kiddy (46). LKB 2117 Multiphor horizontal electrophoresis cell was used (LKB-Prodekter ABS-16125 Bromma, Sweden) (Figure 18). Current was applied to the gel as follows: 100 volts for 10 min before applying the samples to the gel, then 100 volts for 10 minutes after applying the samples to the gel, followed by increasing the voltage to 200 volts for 4 to 5 h.

**Cheese samples**

Cheese samples for electrophoresis were prepared by the procedure of Ledford et al. (49) with slight modification:

Two-tenth of a gram of cheese was minced in mortar and
Figure 16. Groen vacuum condenser used to make UF cheese curd from retentate.
Figure 17. A schematic of UF-cheese curd making.
WHOLE MILK

ADJUSTED TO pH 5.8 WITH CONC. HCL

ULTRAFILTRATION & DIFILTRATION (5X)

RETENTATE

Pasteurized at 63 C for 30 min

Adjusted to pH 5.2 with GDL

Vacuum Evaporated to 39% moisture

UP-CHEESE CURD
Figure 18. LKB 2117 Multiphor horizontal electrophoresis cell used for gel electrophoresis of starter-free Cheddar cheese and UF-cheese curd.
pestle with 0.8 ml distilled water and 2 ml modified Poulík's buffer (35 ml stock Poulík's buffer (0.92% Tris, 1.2% citric acid), 205 ml distilled water, and 174 g urea (46)), then transferred to a 10 ml test tube. Three drops of 1.5 g/ml sucrose solution and 6 drops of dye marker solution (1.5 g amido black + 500 ml of 60% ethanol) (46) were added to the sample. Two drops of mercaptoethanol were added to the mixture 45 min prior to electrophoresis (49).

Staining and destaining the gels

Amido black (2 g amido black, 50 ml glacial acetic acid, 250 ml methanol, and 250 ml distilled water) (46) was used for staining the gels, and gels were destained by several washes with 7% acetic acid (46).

Gel drying

Prior to drying the gels, they were soaked in a preserving solution (30% ethanol, 10% acetic acid, and 10% glycerol) for at least 1 h to prevent cracking during drying. Gels were dried by Bio Rad gel drier, model 244 (Hoefer Scientific Instruments, U.S.A.) (Figure 19).
Figure 19. Bio Rad Gel Drier model 244
Measurement of enzyme concentration

Measurement of enzyme diffusion distance was by scanning in DU-8 a Beckman spectrophotometer. Figure 14 shows the diffusion distance of $10^{-1}$ chymosin unit/ml of chymosin solution in the casein-agar gel, after 48 h incubation at 37 C. The origin (meniscus of casein-agar gel) and the precipitation band are clearly marked. This method of measuring enzyme diffusion distance was as good as using a millivolt recorder, as done by Holmes et al. (40).

Modification of the diffusion technique

Modification of the diffusion technique was first applied to chymosin to predict its accuracy in comparison with the original procedure (40).

Table 1 shows the diffusion distances, the chymosin in whey and curd, and percent of total activity recovered from each sample of freshly coagulated milk. pH of the milk at setting was 6.2. The amount of chymosin added was 3.5 chymosin units/225 g milk. The control represents the original procedure in which the enzyme was extracted from the curd in distilled water, then adjusted to pH 6.8. Extraction of chymosin from the curd by the modified procedure was at pH 6.0 in 1M NaCl solution. The difference
Table 1. Diffusion distances, chymosin concentrations in curd and whey, and total chymosin recovered from 10 replications of freshly coagulated milk.

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<th></th>
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<tr>
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<td>(%)</td>
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<td>(%)</td>
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<td>(%)</td>
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<td>9.6</td>
<td>1.380</td>
<td>39.43</td>
<td>3.69</td>
<td>105.42</td>
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N=10  Mean  2.3+.3  66.2+.8.4  1.2+.33  34.3+.9.6  3.5+.0.2  100.4+.5

df=9  t=.59  t=.59  t=-.45  t=.12

* Represents the original procedure. Enzyme was extracted from curd at pH 6.8.
between the average total chymosin units recovered by the modified procedure and the total chymosin units recovered by the original procedure was 0.025, which is insignificant (Table 1). The average total chymosin units accounted for in curd and whey from 10 replicates was 3.5 chymosin units with a standard deviation of 0.2 which represented 100.43% of the total chymosin added to the milk with a standard deviation of 5.5% (Table 1, Figure 20).

The diffusion distances, amount of enzyme in the curd and whey, and total enzyme retained in samples of freshly coagulated milk for porcine pepsin are shown in Table 2. The pH of milk at setting was 5.8. The pH of enzyme extract for the control was 6.8. Distilled water was used for extraction at a temperature >10°C. The extract was then adjusted to 6.8.

There was a loss of 36.4% of the total porcine pepsin added to milk when extracted by the procedure of Holmes et al (40). This loss was caused by increasing the pH of extraction to 6.8 which inactivated much of the pepsin in the curd. The pH of curd extracted by the modified procedure was 6.0 using 1M NaCl at a temperature <10°C. This allowed complete recovery of all the porcine pepsin from the curd. The average pepsin recovery from five replicates of curd and whey from freshly coagulated milk was 3.58 chymosin units with a standard deviation of 0.12. This represented 102% with a standard deviation of 5.9% of the
Figure 20. Total recovery of chymosin and porcine pepsin in curd and whey from freshly coagulated milk by the original (40) (control) (pH 6.8) and the modified extraction procedure (pH 6.0). Vertical lines at the top of the bars represent standard deviation from the mean.
Table 2. Diffusion distances, porcine pepsin concentrations, and total pepsin recovered from five replications of freshly coagulated milk

<table>
<thead>
<tr>
<th>Sample</th>
<th>Diffusion distance (mm)</th>
<th>Total pepsin recovery (CU)</th>
<th>Total pepsin recovery (%)</th>
<th>Diffusion distance (mm)</th>
<th>Total pepsin recovery (CU)</th>
<th>Total pepsin recovery (%)</th>
<th>Total recovery (CU)</th>
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<td>2.35</td>
<td>2.378</td>
<td>67.94</td>
<td>3.858</td>
<td>110.23</td>
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</table>

N=5, df=4
Mean: 36.7±3.3, 65.6±4, 3.6±0.2, 102±6

* represents the original extraction procedure in which the enzyme was extracted from the curd in distilled water, then adjusted to pH 6.8

+ significant difference at ≤.001

++ significant difference at ≤.01
total porcine pepsin added to the milk.

The distribution of chymosin and porcine pepsin between whey and curd in freshly coagulated milk is shown in Figure 21. Of the total chymosin added to the milk 66.2% with a standard deviation of 8.4% was accounted for in the whey, while 34.27% with a standard deviation of 9.6% was retained in the curd at a setting pH of 6.2 (Table 1). The amount of porcine pepsin retained in curd of freshly coagulated milk set at pH 5.8 was 65.6% with a standard deviation of 4% of the total pepsin added to the milk. The amount of pepsin accounted for in the whey was 36.7% with a standard deviation of 3% of the total porcine pepsin added to the milk. There was more enzyme activity held in curd than when set with chymosin. This is because the pH at setting with chymosin was 6.2, while the pH at setting with porcine pepsin was 5.8.

Porcine pepsin in Cheddar cheese after pressing

Cheddar cheese was made in triplicate from pasteurized whole milk set with porcine pepsin at pH 6.6, 6.4, 6.2, and 6.0. The residual porcine pepsin in the curd after pressing was extracted by the modified procedure and measured by the diffusion technique (40). The amount of porcine pepsin retained in the cheese after pressing depended on the pH of milk at setting (Table 3). When milk was set at pH 6.6 no porcine pepsin was detected in the
Figure 21. Chymosin and porcine pepsin accounted for in curd and whey from freshly coagulated milk by the original (Holmes et al. (40) procedure) and modified extraction procedure. Milk set with chymosin at pH 6.2 and with pepsin at pH 5.8. Vertical lines at the top of the bars represent standard deviation from the mean.
pressed curd, while 5.94% of the pepsin activity added to cheese milk set at 6.0 was retained in the curd after pressing with a standard deviation of 0.3%. At a setting pH value of 6.2 and 6.4, the amount of original pepsin retained in the curd after pressing was 4.79% with a standard deviation of 0.52% and 3.64% with a standard deviation of 0.12%.

Table 3. Percentages of porcine pepsin retained in Cheddar cheese curd after pressing.

<table>
<thead>
<tr>
<th>pH of milk at setting</th>
<th>porcine pepsin accounted for in cheese curd after pressing (%)</th>
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<tr>
<td>6.6</td>
<td>0.00</td>
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<tr>
<td>6.4</td>
<td>3.64 ± 0.12</td>
</tr>
<tr>
<td>6.2</td>
<td>4.79 ± 0.52</td>
</tr>
<tr>
<td>6.0</td>
<td>5.94 ± 0.30</td>
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</table>

Starter-free cheese

Four lots of starter-free cheese milk were adjusted to pH 6.6, 6.4, 6.2, and 6.0 with lactic acid and were set with chymosin or porcine pepsin. Glucono-6-lactone was added to cheese curd after draining the whey, and at salting. The final pH of the curd was 5.1-5.2. The moisture content was 37-39% (75). At different stages during ripening at 7°C, the cheese was examined for degree of proteolysis by polyacrylamide electrophoresis. (Figures 22-31).
Figure 22. Polyacrylamide gel electrophoretic patterns of starter-free Cheddar cheese made from milk set with chymosin at pH 6.6. (Pattern A: after pressing, pattern B: 10 days old, and pattern C: 12 weeks old).
Figure 23. Polyacrylamide gel electrophoretic patterns of starter-free Cheddar cheese made from milk set with porcine pepsin at pH 6.6. (Pattern A: after pressing, pattern B: 10 days old, pattern C: 6 weeks old, pattern D: 10 weeks old, and pattern E: 14 weeks old)
Figure 24. Polyacrylamide gel electrophoretic patterns of starter-free Cheddar cheese made from milk set with porcine pepsin at pH 6.4. (Pattern A: after pressing, pattern B: 10 days old, pattern C: 14 weeks old).
Figure 25. Polyacrylamide gel electrophoretic patterns of starter-free Cheddar cheese made from milk set with porcine pepsin at pH 6.2. (Pattern A: after pressing, pattern B: 10 days old, pattern C: 12 weeks old).
Figure 26. Polyacrylamide gel electrophoretic patterns of starter-free Cheddar cheese made from milk set with porcine pepsin at pH 6.0. (Pattern A: after pressing, pattern B: 10 days old, pattern C: 12 weeks old).
Figure 27. Polyacrylamide gel electrophoretic patterns of starter-free and coagulant-free UF cheese curd after 1 week ripening at 7°C (pattern A), and starter-free Cheddar cheese of the same age made from milk set with porcine pepsin at pH 6.6 (pattern B).
Figure 28. Polyacrylamide gel electrophoretic patterns of starter-free and coagulant-free UF cheese curd after 6 weeks ripening at 7 C. (pattern A), and starter-free Cheddar cheese of the same age made from milk set with porcine pepsin at pH 6.6 (pattern B).
Figure 29. Polyacrylamide gel electrophoretic pattern of starter-free and coagulant-free UF cheese curd after 14 weeks ripening at 7 °C.
Figure 30. Polyacrylamide gel electrophoretic pattern of starter-free Cheddar cheese made from milk set with crystalline porcine pepsin at pH 6.6 after 12 weeks ripening at 7 C.
Figure 31. Polyacrylamide gel electrophoretic pattern of starter-free Cheddar cheese made from milk set with crystalline porcine pepsin at pH 6.0 after 14 weeks ripening at 7 C.
**Milk set at pH 6.6 with chymosin**

Figure 22 shows the polyacrylamide gel electrophoretic patterns of starter free Cheddar cheese. The milk was set with chymosin, and acidified with lactic acid and glucono-δ-lactone. Pattern A represents the cheese after pressing. There was some initial degradation in the α_{s1}-casein band which continued to degrade as the cheese ripened to 10 days (pattern B), and 12 weeks (pattern C). Some degradation of β-casein also was evident after 12 weeks as shown in pattern C.

**Milk set at pH 6.6 with porcine pepsin**

Figure 23 shows the polyacrylamide gel electrophoretic patterns of Cheddar cheese set at pH 6.6 with porcine pepsin and acidified with lactic acid and glucono-δ-lactone (no starter). Pattern A shows that after pressing, there was no breakdown in α_{s1}-casein. A slight degradation of α_{s1}-casein appeared after 10 days ripening as shown in pattern B. Pattern C shows more breakdown of α_{s}-casein after 6 weeks ripening. Degradation of α_{s1}-casein continued as the age of cheese increased to 10 and 14 weeks as shown in patterns D and E.

**Milk set at pH 6.4 with porcine pepsin**

Figure 24 shows the polyacrylamide electrophoretic
patterns of Cheddar cheese made from milk set at pH 6.4 with porcine pepsin, and acidified with glucono-δ-lactone and lactic acid. A breakdown in αs1-casein appeared in pattern A which represents the cheese after pressing. Pattern B shows the breakdown in αs1-casein after 10 days ripening. After 14 weeks ripening more proteolysis occurred in αs1-casein and slight proteolysis in β-casein.

**Milk set at pH 6.2 and 6.0 with porcine pepsin**

Figures 25 and 26 show the polyacrylamide gel electrophoretic patterns of Cheddar cheeses made from milk set with porcine pepsin set at pH 6.2 and 6.0 respectively, and acidified with lactic acid and glucono-δ-lactone (no starter).

In cheese set at pH 6.2 (Figure 25), a clear breakdown in the αs1-casein occurred after pressing as is shown in pattern A. After 10 days ripening the αs1-casein was highly degraded (pattern B). More degradation in αs1-casein and some degradation in β-casein appears in pattern C which represents cheese at 12 weeks of age.

The electrophoretic patterns of Cheese set with porcine pepsin at pH 6.0 are shown in figure 26. Cheese after pressing, 10 days, and 12 weeks ripening are represented by electrophoretic patterns A, B, and C. The degradation
in $\alpha_{s1}$-casein started immediately after pressing, and increased markedly as the cheese aged.

**Starter-free and coagulant-free cheese curd (UF cheese curd)**

Cheese curd was made from ultrafiltered milk (26) with a final pH of 5.2, and a moisture of 39%. Milk was adjusted to pH 5.8 with HCl prior to ultrafiltration and difiltration (5X). The retentate was reduced to pH 5.2 with glucono-$\delta$-lactone prior to evaporation to 39% moisture (no starter and no milk coagulant). The amount of glucono-$\delta$-lactone required to drop the pH of the retentate to 5.1-5.2 was 50 g/1, and allowed to act for 3 h at 30 C. After six weeks ripening, the standard plate count of the cheese was $7\times10^3$ per g, and after 14 weeks, it was $55\times10^3$ per g. Electrophoretic patterns of the UF cheese curd are shown in Figures 27, 28, and 29.

Figure 27 A represents 1 week old UF cheese curd. Pattern B of the same figure represents Cheddar cheese 1 week of age set with porcine pepsin at pH 6.6 and acidified with lactic acid and glucono-$\delta$-lactone (no starter). Both patterns show very faint degraded band of $\alpha_{s1}$-casein.

Pattern A of Figure 28 represents 6 week old UF cheese curd (no starter and no coagulant) which shows a degradation in the $\alpha_{s1}$-casein and beta casein. Degradation in
cheese set with porcine pepsin at pH 6.6 (no starter) of the same age (pattern B). The same UF cheese curd after 14 weeks ripening is shown in Figure 29. Substantial degradation of $\alpha_{s1}$-casein is evident.

Milk set with crystalline porcine pepsin at pH 6.6 and 6.0

Crystalline porcine pepsin was used to make starter-free cheese set at pH 6.6 and 6.0. The polyacrylamide gel electrophoretic pattern of cheese 12 weeks of age set at pH 6.6 is shown in Figure 30. A clear degraded band of $\alpha_{s1}$-casein is evident. The polyacrylamide gel electrophoretic patterns of cheese 14 weeks of age, set with crystalline porcine pepsin (no starter) at pH 6.0 is shown in Figure 31. There was very excessive degradation of $\alpha_{s1}$-casein. A breakdown in $\beta$-casein is clearly evident.
DISCUSSION

A modification of the extraction procedure of Holmes et al (40) diffusion technique was based on the assumption that the pH dependent interaction between cheese curd and chymosin or porcine pepsin was ionic. Such an interaction can be weakened by extraction with sodium chloride solution instead of water. This could enable extraction of these enzymes at pH values harmless to porcine pepsin. Extraction in 1M NaCl at pH 6.0 was applied first to chymosin and was found to be as effective as that of the original procedure. A complete recovery of chymosin from curd was accomplished. The modified extraction procedure was next applied to porcine pepsin curd, which also allowed complete recovery of the enzyme from curd formed from freshly coagulated milk at pH 6.0.

The modified extraction procedure for measuring low concentrations of milk clotting enzymes allowed us to answer the question as to whether porcine pepsin survives the Cheddar cheese making process. The amount of porcine pepsin retained in Cheddar cheese curd after pressing depended upon the pH of the milk at setting. This agrees with other workers (70). The lower the pH at setting the more porcine pepsin was retained in Cheddar cheese. However, at a setting pH of 6.6 no porcine pepsin was
detected in Cheddar cheese curd after pressing. This means that a complete destruction of the enzyme occurred during the manufacture of Cheddar cheese set at pH 6.6.

A breakdown in $\alpha_{S1}$-casein occurred during the ripening of Cheddar cheese set with porcine pepsin at pH 6.6 (without starter) even though no porcine pepsin survived the cheese making process (Figure 23). O'Keeffe et al. (70) claimed that some porcine pepsin survived Cheddar cheese making even though the pH of setting was 6.6 or 6.65.

The cause of $\alpha_{S1}$-casein degradation in the absence of pepsin was investigated. Cheese curd was made from ultra-filtered milk acidified with hydrochloric acid and glucono-$\delta$-lactone to a pH of typical Cheddar cheese after pressing (pH 5.2). The acidified retentate was condensed to 39% moisture under vacuum. No milk coagulant and no starter was employed. This UF-curd contained about 4% whey protein as compared to regular curd that contained approximately 0.5%. However whey proteins are much more resistant to proteolysis than the caseins, and it is unlikely that this difference would have interfered with the results of this study.

The electrophoretic patterns of the UF-cheese curd after 1 week, 6 weeks and 14 weeks ripening appear similar to those of Cheddar cheese of the same age, set with porcine pepsin at pH 6.6 (no starter). (Figures 27, 28,
The degradation of $\alpha_s$-casein in UF-cheese curd and Cheddar cheese curd set with porcine pepsin at pH 6.6 must have been caused by factors other than pepsin, starter bacteria, or contaminating bacteria, since the total bacterial count of the cheese curd after 6 weeks ripening was only 7000 per g. The factor that caused the breakdown in $\alpha_s$-casein could have been an acid milk protease which survived the pasteurization process. This protease has an optimum pH of 4 (43). We agree with Visser et al. (92), who found that milk protease caused some degradation in $\alpha_s$-casein and $\beta$-casein in aseptic Gouda cheese that was free of milk coagulant and starter bacteria.

The degradation of $\alpha_s$-casein in Cheddar cheese set with porcine pepsin (no starter) increased as the pH at setting decreased from 6.4 to 6.0 (Figures 24, 25, and 26) because progressively more porcine pepsin was retained in cheese curd. This is in agreement with other workers (40). Polyacrylamide gel electrophoretic patterns of Cheddar cheese set with chymosin (no starter) at pH 6.6 showed a breakdown in $\alpha_s$-casein after pressing (Figure 22). However, the extent of degradation in $\alpha_s$-casein after pressing, 10 days and 12 weeks ripening is about the same as in cheese of similar ages that set with porcine pepsin at pH 6.2 (no starter). The amount of chymosin that was retained in Cheddar cheese curd after pressing was about 6% of the total chymosin activity added to the milk (40).
The amount of porcine pepsin retained in cheese curd set at pH 6.2 was 4.7%. At pH 6.0, 6% of the total pepsin added to cheese milk was recovered in cheese curd after pressing. Crystalline porcine pepsin was used in this study to compare its proteolytic effect on caseins with that of crude pepsin. Cheddar cheese was made from milk set with crystalline porcine pepsin at pH 6.6 and 6.0 acidified with lactic acid and glucono-δ-lactone (no starter). The cheese set at pH 6.6 had about the same proteolytic breakdown in α₃₁-casein as that made with crude porcine pepsin (Figures 23 and 30). The electrophoretic pattern of cheese set at pH 6.0 with crystalline porcine pepsin showed more degradation in α₃₁-casein and β-casein after 12 weeks of ripening than cheese of the same age set at the same pH with crude porcine pepsin (Figures 26, 31).

Further studies are needed to determine the effect of starter proteinases on caseins of Cheddar cheese by making coagulant free cheese curd acidified with starter bacteria. The degradation of caseins during ripening caused by starter bacteria could be determined by polyacrylamide gel electrophoresis, and compared with the starter free Cheddar cheese set with chymosin and porcine pepsin. The degraded bands could be determined quantitatively by measuring their molecular weight by SDS-electrophoresis.
Another approach to make coagulant-free cheese was proposed by Visser (88). The first action of chymosin was performed on Ca\(^{++}\) depleted milk. The coagulant was then inactivated by pasteurization at 72 °C for 15 sec. Calcium chloride was then added at 4-5 °C, followed by dielectric heating to 30 °C to induce coagulation. This kind of cheese could be compared electrophoretically and organoleptically with normal and starter-free Cheddar cheese.
CONCLUSIONS

1. Porcine pepsin survival during Cheddar cheese making depends upon the pH of milk at setting. The lower the pH the more porcine pepsin was retained in the curd. No pepsin activity was detected in cheese when the pH at setting was 6.6.

2. Degradation of $\alpha_{S1}$-casein during the curing of Cheddar cheese made with porcine pepsin increased as the pH at setting decreased.

3. Degradation of $\alpha_{S1}$-casein occurred even though no porcine pepsin or starter bacteria were present in cheese curd. This might be caused by the milk proteases.

4. When Cheddar cheese was set at pH 6.6, porcine pepsin did not survive the Cheddar cheese making process, therefore, could not contribute to cheese curing.

5. Porcine pepsin had greater proteolytic activity on casein than chymosin when equal amount of both enzymes were retained in Cheddar cheese curd.
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