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INFLUENCE OF VARIOUS COAGULANTS ON CASEIN DEGRADATION IN AMERICAN CHEDDAR TYPE CHEESE

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ABSTRACT

INFLUENCE OF VARIOUS COAGULANTS ON CASEIN DEGRADATION IN AMERICAN CHEDDAR TYPE CHEESE

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Using six commercial milk coagulants, twenty-four batches of American Cheddar-type cheese were prepared and ripened for 9 months. No significant differences in cheese yield or composition were related to the type of coagulant.

The type of coagulant significantly affected the levels of pH 4.6-soluble nitrogen after 3, 6, and 9 months of ripening. Soluble nitrogen (in 12% TCA) was significantly affected after 9 months. Cheese made with Actinomucor pusillus protease and with a blend of Actinomucor pusillus protease and bovine pepsin showed the highest levels of the soluble nitrogen. Intermediate values were observed for the cheese made with calf rennet or with bovine pepsin. The lowest levels were for cheese made with a thermosensitive Actinomucor miehei protease or with a blend of calf rennet and porcine pepsin.

Discontinuous polyacrylamide gel electrophoresis indicated that when the enzymes of animal origin were used, α_{S1}-casein was degraded, first to a major zone of higher electrophoretic mobility. β-Casein was practically

unaffected. When microbial enzymes were used, α_{S1}-casein was degraded to a greater number of electrophoretically discernable zones. Like animal coagulant action, β-casein was less resistant to microbial coagulant action. SDS-Polyacrylamide gel electrophoresis indicated a relatively greater number of products of lower molecular weight formed in the cheeses made with the microbial enzymes.

Sensory evaluation indicated no significant differences in flavor due to the coagulant used. The cheese made with the calf rennet/porcine pepsin blend received the highest score followed by the cheese made with Actinomucor miehei protease. The cheese made with the blend of Actinomucor pusillus protease and bovine pepsin was the only one with significant bitterness throughout ripening.

Measurements of residual coagulant indicated that calf rennet was retained more in cheese than the microbial enzymes. The blend of calf rennet and porcine pepsin was retained less than pure calf rennet. Bovine pepsin and the blend containing bovine pepsin and Actinomucor pusillus protease were retained in curd more than any other coagulant studied.

To my mother and to Jim.

I love you.

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INTRODUCTION

Until recently calf rennet has been the traditional coagulant used in cheese manufacture. However, an increase in world-wide cheese production together with a decrease in the number of good quality calves' stomachs (vells) have affected its price. These factors led to an active search for suitable substitutes offering a comparative price advantage over the traditional calf rennet coagulant.

Presently, six acid proteases are used as rennet substitutes in commercial cheese manufacture. They are: bovine, porcine and chicken pepsins, and extracellular proteases from Actinomucor miehei (formerly Mucor miehei), Actinomucor pusillus (formerly Mucor pusillus), and Endothia parasitica.

Rennet substitutes differ from calf rennet in proteolytic specificity. As a consequence, more intense or less specific breakdown in the cheese casein may occur causing defects in the body and flavor of the finished product. Several reports in the literature cover technological aspects of selection of type of coagulant and optimum conditions for maximum cheese quality. Also available are a number of studies describing the action of these enzymes on whole casein and its fractions.

This work was directed toward understanding the relationship between hydrolysis of casein by commercial coagulants and organoleptic quality of cheese. Accordingly, American-Cheddar-type cheese was made with a number of commercial milk coagulating enzyme preparations recently introduced in the market. The development of cheese flavor and bitterness was investigated as a function of time of ripening. The work includes monitoring of protein degradation during ripening by determination of soluble nitrogen fractions in the cheese, gel electrophoretic analysis for measurement of peptides formed, and studies of some of the clotting and proteolytic properties of the different coagulants used.

LITERATURE REVIEW

Need for Coagulants: Historical Perspective

Although the origin of cheese is uncertain, it is agreed that it was first produced by nomadic tribes in the Middle East (Scott, 1973; Eekhof-Stork, 1976; Kosikowski, 1978). The first curds might have resulted from the precipitation of the proteins in milk at their isoelectric point due to lactic or other acids produced by microorganisms. It is probable that sometime later, the use of animal skins and stomach pouches for carrying surplus milk led to the use of rennin or chymosin in the milk to produce curds.

Traditionally, in the manufacture of Cheddar cheese, milk is coagulated at a pH above the isoelectric point of casein by rennin or chymosin (E.C. 3.4.23.3), an acid protease extracted from the mucosa of the fourth stomach (abomasum) of the young calf. The enzyme preparation called rennet is used as a crude extract, paste or powder.

In the last 20 years, world production of cheese has been increasing at an annual growth rate of 8.3% and it stands now at about 9.18 billion kg. Western European countries are very large producers of cheese and in 1978 produced approximately 47.5% of all the cheese in the

world. Northern American countries, including the United States, Canada, and Mexico, produced 22.4%. Eastern Europe, including the Soviet Union, produced about 18% of the world's supply. South America produced about 6.32%, and Oceania, including Australia and New Zealand, about 2.36% (Christensen, 1979). This increased demand for cheese has been followed by a steady decline of good quality calves' stomachs. For example in the U.S.A., the number of vells decreased from a high of 13 million in 1960 to a low of 2 million in 1973. They increased slightly to 4.5 million in 1976, but have decreased each year since. Therefore, in the last 15 years a major trend in the dairy industry has been to use a variety of milk coagulating enzymes of both animal and microbial origin (Nelson, 1975).

The six sources of milk clotting enzymes and their blends in commercial use in the United States are shown in Table 1. The three fermentation-derived enzymes had a history of use in other countries prior to clearance from The Food and Drug Administration (FDA). The FDA regulation that covers fermentation-derived milk clotting enzymes (Anonymous, 1975) was amended in 1967, 1969 and 1972 to permit the use of Endothia parasitica, Actinomucor pusillus and Actinomucor miehei proteases, respectively.

Table 1. Milk clotting enzymes utilized in the United States.[a,b]

A. Ruminant Animal - Derived Enzymes	
	Calf Rennet (85-95% chymosin, 5-15% bovine pepsin)
	Bovine Rennet (55-60% bovine pepsin, 40-45% chymosin)
B. Single Stomach Animal - Derived Enzymes	
	Porcine Pepsin
C. Fermentation - Derived Enzymes	
	Endothia parasitica Protease
	Actinomucor miehei Protease
	Actinomucor pusillus Protease
D. Milk Clotting Enzyme Blends	
	Calf Rennet/Porcine Pepsin (40-45% chymosin, 50% porcine pepsin, 5-10% bovine pepsin)
	Bovine Rennet/Porcine Pepsin (20-25% chymosin, 40-45% bovine pepsin, 30-40% porcine pepsin)
	Actinomucor miehei Protease/Porcine Pepsin
	Actinomucor pusillus Protease/ Porcine Pepsin
	Actinomucor pusillus Protease/Bovine Pepsin

[a]Nelson, 1975.

[b]Sellars, 1982.

Coagulation of Milk

The early attempts to explain the clotting of milk by rennin were based on the belief that casein was homomolecular (Phelan, 1977). It was proposed that rennin converted the native casein into a new form called paracasein, which clotted in the presence of calcium ions. Hammarsten's idea may be represented as:

$$\text{casein} \xrightarrow{\text{(rennin)}} \text{paracasein} + \text{soluble whey albumin}$$

$$\text{paracasein} \xrightarrow{Ca^{++}} \text{insoluble clot.}$$

Further studies of the casein revealed that it is not a simple but a complex protein containing a range of components of which α_s-, β- and κ-caseins are major fractions. These components are determined according to the genetic type of the ruminant (lyster, 1972; Whitney, Brunner, Ebner, Farrel, Josephson, Morr and Swaisgood, 1976). Gamma casein is now known to be derived from β-casein hydrolysis by native milk protease (Brunner, 1981).

In 1958, Waugh reported that κ-casein is the only protein in the casein micelle altered during coagulation. Since then, the central role of κ-casein has been extensively studied. It is now generally accepted that the action of rennin involves three stages (McKinlay and Wake, 1971; Ernstrom and Wong, 1974; Yamamoto, 1975; Phelan,

1977; and Kosikowski, 1978. In the first stage, an enzymatic phase, rennin cleaves the phenylalanine-methionine bond located in positions 105 and 106 of the amino acid sequence of κ-casein. This destroys κ-casein's stabilizing action on calcium sensitive α_s-caseins, and β-casein. The result is para κ-casein and caseinomacropeptide.

$$\kappa\text{-casein} \xrightarrow{\text{(rennin)}} \text{para } \kappa\text{-casein} + \text{caseino-macropeptide}$$

Para κ-casein is a basic, hydrophobic moiety. The macropeptide is highly acidic, containing phosphorus, and a variable amount of carbohydrate. Para κ-casein constitutes about 30% of the κ-casein monomer.

In the second stage, a non enzymatic phase, the resulting para κ-casein precipitates in the presence of calcium ions as dicalcium para κ-casein. The α_s-caseins and the β-casein for whom intact κ-casein had served as a protective colloid are no longer stabilized; in direct contact with calcium, these proteins in combination with para κ-casein are removed from suspension to form the curd. A third non-specific enzymatic stage can be considered during the ripening of the cheese when caseins constituting the curd are gradually degraded.

Electron micrographs show that in the first stage, before aggregation occurs, rennet does not affect the

structure of the casein micelle (Green, 1980). Rennet seems to reduce the electrophoretic mobility of the micelle due to reduction of the surface negative charge caused by the conversion of κ-casein to para κ-casein. Green, Hobbs and Morant (1978) and Green, Hobbs, Morant and Hill (1978) studied the aggregation of micelles by viscometry and electron microscopy. The increase in viscosity of rennet-treated milks was considered a measure of aggregation of the casein micelle. Aggregation did not start until the enzymic phase was almost complete, i.e. at close to 60% of the visually observed clotting time in normal milk at 30 C. Small chains formed first followed by assembly into a network. Eventually, the network consisted of loose chains about five micelles thick separated by wide spaces. The loose packing of the micelles enabled much serum to be trapped. Not all micelles, however, were bound to the network.

The Hydrolysis of the Caseins

Aside from the studies on the cleavage of a single bond in κ-casein by rennin the action of this protease on other casein fractions has been investigated. Creamer, Mills and Richards (1971) studied the effect of crystalline rennin and rennet extract on β-casein-B by discontinuous electrophoresis with quantitation by densitometry. As the reaction proceeded the quantity of β-casein decreased while

a more mobile component, B-I, increased. In time B-I slowly disappeared and two other components, B-II and B-III, appeared. B-III apparently originated from B-II. This pattern of breakdown was observed in β-casein in isolated form but was not seen in cheese. This observation was not clarified by the authors. Previously, Ledford, O'Sullivan and Nath (1966) and Edwards and Kosikowski (1969) had shown that in Cheddar cheese α_{S1}-casein was extensively degraded while β-casein underwent very little, if any, proteolysis. In later work, Creamer (1976) showed that the rate of hydrolysis of the β-casein and the peptides was dependent on NaCl concentration or pH at high temperatures. He concluded that in a system such as cheese where the casein is at high concentration and the NaCl concentration is also high, β-casein exists as a self-associated polymer or, complexed with α_{S1}-casein. Thus, rennin sensitive bonds are not accessible to the enzyme. In the same study, Creamer isolated the peptides previously designated B-I, B-II and B-III. Their major constituents were the residues 1-189, 1-166 or 164 and 1-139 of β-casein, respectively.

In a previous study, Fox and Walley (1971) had investigated the influence of NaCl on casein proteolysis by rennin and pepsin using a gel electrophoretic method. They found that the proteolysis of β-casein by these enzymes was completely inhibited in the presence of 10% NaCl and

significantly reduced by 5% NaCl. For α_{S1}-casein, the rate of proteolysis was maximal in the presence of 5-10% NaCl, thus supporting the assumptions of Creamer.

Further investigations on the various factors which influence the susceptibility of β-casein in Cheddar cheese proved that NaCl content selectively inhibited the breakdown of β-casein (Phelan, Ginney and Fox, 1973). Therefore, β-casein resistance to proteolysis in Cheddar cheese could be due to polymerization, or possibly a consequence of conformational change in the protein molecules favored by the relative small decrease in water activity produced by NaCl.

Creamer (1975) also studied the products of β-casein breakdown in Gouda and Cheddar cheese. He examined the high molecular weight peptides of low electrophoretic mobility formed during ripening. He found that with increased ripening time the quantity of α_{S1}-casein decreased with a corresponding increase in α_{S1}-I casein. Also, an increase in low mobility peptides was found. The Cheddar cheese showed more degradation of α_{S1}-casein and less formation of low mobility peptides than the Gouda cheese. These low mobility peptides were shown to be identical to TS-, R- and γ-casein. These caseins are derived from β-casein (Gordon, Grover, Greenberg, Jones, Kalan, Peterson and Towned, 1972). Since previous reports (Creamer et al., 1971; Phelan et al., 1973) indicated that

in cheese β-casein is resistant to rennin attack, it was unlikely that rennin was responsible for the formation of these peptides. Creamer suggested that the most likely source was the natural alkaline protease. It was not clear why β-casein in Cheddar cheese was degraded slower than in Gouda cheese. Creamer suggested, however, that the higher pH and moisture content in Gouda cheese might explain this difference.

Moreno and Kosikowski (1972) reported a protease with maximum activity toward β-casein at pH 5.5 and temperatures of 22 C and 30 C. Most of the peptides formed were bitter. They suggested that if this protease hydrolyzes β-casein, the peptides formed early in the degradation would likely impart a bitter taste to cheese. Further hydrolysis would liberate amino acids which would be transformed by other enzymatic systems in cheese to acids, aldehydes, and amines. These products would cause a definite aromatic flavor in cheese. At low temperatures the protease would not be expected to contribute significantly to the ripening of the cheese, but at 22 C it could do so.

Another study on the activity of the natural alkaline milk protease in raw and pasteurized milk indicated that β-casein is degraded two or three times faster than α_{S1}-casein (Noomen, 1975). α_{S1}-casein is known to be more susceptible to proteolysis by rennet than β-casein (Ledford et al., 1966; Edwards and Kosikowski, 1969). Mulvihill and

Fox (1977b) found that α_{S1}-casein apparently had six-bonds susceptible to rennet. The specificity of the enzyme on α_{S1}-casein depended on the pH of the reaction and on the state of aggregation of the substrate. Therefore, at pH 5.8 α_{S1}-casein was hydrolyzed to α_{S1}-I; at pH values higher than 5.8, α_{S1}-I was hydrolyzed to yield α_{S1}-II, α_{S1}-III and α_{S1}-IV. This occurred in the presence or absence of urea. At their isoelectric points, α_{S1}-II, α_{S1}-III and α_{S1}-IV were not formed in the absence of urea; instead, α_{S1}-V was produced. This is probably because with aggregation some bonds are unavailable to hydrolysis. When urea was used for dissociation at this pH, α_{S1}-II, α_{S1}-III and α_{S1}-IV were formed together with another peptide, α_{S1}-VI.

Para κ-casein is hydrolyzed only very slowly by rennin; α_{S2}-caseins are also very resistant and the hydrolysis of the δ-caseins has not been investigated (Fox, 1981).

Role of Whey Proteins in Rennin Action

After heating milk there is a reduction in the total amount of peptides released by the action of rennin (Wilson and Wheelock, 1972). This inhibition of the primary phase of rennin action is probably due to complex formation between β-lactoglobulin and a portion of κ-casein, so that κ-casein cannot be hydrolyzed by rennin. Wheelock and Kirk

(1974) confirmed this assumption by using resuspended casein micelles to which β-lactoglobulin was added during heating. The same co-workers later showed by using sulfhydryl blocking agents that the inhibition of the number of peptides released by rennin is due to sulfhydryl interaction between κ-casein and β-lactoglobulin, followed by complex formation (Wilson, Wheelock and Kirk, 1974).

Since α-lactalbumin also contains disulfide groups, Shalabi and Wheelock (1976) investigated the possible complex formation between α-lactalbumin and κ-casein. Their results demonstrated that the inhibition of the primary phase of rennin action on the casein can also be caused by the presence of α-lactalbumin during heating. Less peptides were released with β-lactoglobulin. This suggested that there may be a limited scope for interaction between κ-casein and α-lactalbumin.

Rennet Substitutes

Nearly all proteolytic enzymes will clot milk under proper conditions. Therefore, they have been obtained from virtually every class of living organism (Ernstrom and Wong, 1974). Preparations from plant juices and from bacteria have been used in cheesemaking in India and other countries where there are objections to the slaughtering of animals (Berridge, 1954). A large number of enzymes have been screened but just a few seem to be appropriate.

Since no significant differences in the nutrient composition of the cheeses can be attributed to the type of clotting enzyme (Wong, Lacroix, Vestal and Alford, 1977), the main problem is the intense breakdown in the curd caused by the coagulant. According to Phelan (1977), rennet substitutes used in cheese making must meet a number of criteria:

1. high clotting to proteolytic activity ratio.
2. minimal activity of contaminating enzymes.
3. minimal content of pathogens and gas formers.
4. no toxicity and no antibiotic activity.
5. reasonable shelf-life and ease of handling.
6. no additional changes in cheese making.
7. no adverse effect on the flavor, body, texture and yield of the finished product.
8. thermostability comparable to that of rennet.

Rennet Substitutes of Animal Origin

The stomachs of animals other than calf have received increased attention in recent years. Green (1972) found that Cheddar cheese made with porcine pepsin had poorer organoleptic quality than the cheese made with calf rennet. Fruity flavor was the most noticeable defect. The cheese made with porcine pepsin had harder body, developed flavor slowly and required longer aging than cheese made with calf rennet. Apparently this is due to slow proteolysis resulting from inactivation of the enzymes

during cheese manufacture (Green and Foster, 1974; Green, 1977; Thunell, Duersch and Ernstrom, 1979).

Ma and Nakai (1980) chemically modified the carboxyl groups in porcine pepsin by carboimide-mediated amine formation. The heat stability of the enzyme was improved. The authors suggested that the carboxyl-modified pepsin might be a better milk coagulant than the native enzyme since a larger amount of the active enzyme is retained in the curd. This accelerates the ripening of the cheese and reduces the cost of production.

Using Weizmann's chicken enzyme Gutfeld and Rosenfeld (1975) reported the production of cheese of nearly equal quality to cheese manufactured with calf rennet. They claimed higher quality to that of cheeses produced with microbial rennets: Actinomucor miehei, Actinomucor pusillus and Endothia parasitica. Chicken pepsin prepared by Husek and Dedek (1981) was used for cheesemaking and it proved to have a more pronounced and less specific casein proteolysis than calf rennet. Part substitution (30%) of calf rennet with chicken pepsin, however, produced no changes in quality of Edam, Emmenthal, and Bel Paese cheese.

Anifantakis and Green (1980) described a method for the extraction of milk coagulating enzymes from lamb's and kid's abomasa. Greek cheesemakers prefer these enzymes to calf rennet because they impart a peppery flavor to the cheese, presumably due to the action of the lipases.

Eino, Diggs, Irvine and Stanley (1976) examined cheese curds obtained with calf rennet, bovine pepsin and porcine pepsin by scanning electron microscopy. They observed that the curds made with bovine rennet and porcine pepsin were similar in structure and orientation. The curd produced with calf rennet had a more compact and organized structure.

Rennet Substitutes of Vegetable Origin

Enzymes from higher plants have been traditionally used for curd production in certain areas of the world on religious grounds when calf rennet is not acceptable. Unlike the substitutes of animal and fungal origin, the majority of the vegetable proteases are unsuitable for cheese production due to their potent proteolytic activity which results on digestion of the curd. Eskin and Landman (1975) purified and characterized an enzyme from the juice of ash gourd (Benincasa cerifera). The enzyme was used for Cheddar cheese manufacture (Gupta and Eskin, 1977); some changes in the manufacturing process were required. The quality evaluation indicated that the cheese was highly acceptable, receiving an overall rating only slightly lower than that of cheese produced with calf rennet. The shorter ripening period required was considered a potential economic advantage of this enzyme.

The coagulating enzymes from the green and ripened berries of Solanum torvun were extracted (Hamdy, Cheded, El-Koussy and Foda, 1976) and used for manufacture of Domiati cheese (El-Koussy, Cheded, Foda and Hamdy, 1976). The sensory evaluation indicated quality comparable to cheese made using calf rennet. Also, the yield was slightly higher.

Recent studies by Tavasolian and Shabbak (1979) showed the presence of a milk coagulating enzyme in the defatted meal of safflower seeds. The authors obtained a sweet, soft, creamy white curd. The high quantity of defatted meal available as a byproduct of oil production was pointed out as an economic advantage of this enzyme source.

Rennet Substitutes of Bacterial Origin

Some investigators have reported successful use of bacteria-derived proteases for cheese manufacture. However, a large body of evidence points out the excessive proteolysis in cheeses made with these enzymes. Phelan (1973) prepared cheese with a protease from Bacillus polimixa and obtained a cheese of poor quality. He concluded that it was probably due to preferential degradation of β-casein. The same reason was given by Zvyagintsev, Sergeeva and Gudkov (1971) for the poor quality of ripened cheeses prepared with proteases from Bacillus subtilis and Bacillus polimixa.

Since the bacterial substitutes show a higher proteolytic activity than rennet while porcine pepsin shows lower activity, Husek and Teply (1978) assessed the effectiveness of a mixture of these two enzymes. A slower proteolysis and a higher specificity in the degradation of α_{S1}- and β-casein was achieved. Consequently, they recommended the use of the mixture in the manufacture of soft cheeses. Krishna Rao and Mathur (1979) prepared Cheddar cheese using a 50:50 mixture of enzyme from Bacillus subtilis K-26 and calf rennet. They found that the experimental cheese was as acceptable as the control cheese.

Substitutes of Fungal Origin

A number of fungal proteases have been investigated: proteases from Aspergillus nidulans, Aspergillus glaucus, Symcephalastrum racemosun, Cladosporium herbarum, Bysochlamus fulva, Aspergillus candidus, Aspergillus niger, Rhizopus oligosporus and others.

The proteases from Endothia parasitica, Actinomucor pusillus var. lindt and Actinomucor miehei have been accepted as "safe and suitable" substitutes for calf rennet in the manufacture of cheese in the United States (Ernstrom and Wong, 1974; Nelson, 1975; Prager, 1977). These enzyme preparations were widely investigated in recent years. Martens and Naudts (1976) have extensively

reviewed these coagulants. Some researchers claim that there is no difference between cheese made with fungal enzymes and cheese made with calf rennet. Others, however, noted a bitter flavor in the cheese made with the fungal preparations. This is commonly attributed to a more extensive breakdown of casein by these enzymes.

Vanderpoorten and Weckx (1972) compared the proteolytic properties of enzyme preparations from calf rennet, Actinomucor miehei, Actinomucor pusillus and Endothia parasitica in casein solutions. The microbial rennets liberated more non-protein nitrogen (NPN) from whole casein and from α_{S1}- and β-fractions than did calf rennet. The Endothia parasitica preparation was especially proteolytic. By electrophoresis, it was shown that the Actinomucor preparations had a stronger proteolytic activity against α_{S1}-casein than did calf rennet. Both α_{S1}- and β-casein were extensively degraded by the Endothia parasitica preparation. In general, it appeared that α_{S1}- and β-casein gave a characteristic pattern for each of the coagulating enzymes. When electrophoresis was performed on Gouda cheeses made with the different preparations, the differences in the electrophoretic pattern were particularly pronounced in the region of the α_{S1}-casein. The cheese made with the Endothia parasitica preparation also showed clear degradation of β-casein. These results are in agreement with those of Mickelsen and Fish (1970).

More evidence of the greater hydrolytic capacity of the Endothia parasitica protease on whole, α_{S1}-, and β-casein was given by Tam and Whitaker (1972). They measured spectrophotometrically the number of peptide bonds hydrolyzed after reaction with 2, 3, 4 trinitrobenzene sulfonic acid. According to Vamos-Vigyazo, El-Hawary, and Kiss (1980) differences between the proteolytic activity of Endothia parasitica protease and calf rennet occur only if the protein has been considerably denatured during purification. This may be one of the causes for the discrepancies found in the literature. These differences in activity may be unimportant during practical cheese-making.

El-Shibiny and El-Salam (1976) studied the action of different fungal proteases on β-casein from cow's and buffalo's milk. The order of hydrolytic activity was: Endothia parasitica, Actinomucor pusillus, Actinomucor miehei proteases, and calf rennet. In all cases the activity was higher towards buffalo's milk. The action of the same enzymes on α_{S1}-casein from cow's and buffalo's milk was also investigated (El-Shibiny and El-Salam, 1977). The rate of hydrolysis was almost the same for cow's and buffalo's α_{S1}-casein, but the degradation products were not identical for the two caseins. The order of hydrolytic activity was now Endothia parasitica, Actinomucor miehei, Actinomucor pusillus and calf rennet.

Jasmul, Reps, Poznanski and Zelazowska (1982) combined porcine pepsin and fromase (Actinomucor pusillus var. Lindt preparation) in the manufacture of Edam and Kortowski cheese. The most efficient proportions were 50/50 or 70/30 for Edam cheese and 85/15 for Kortowski cheese. Green and Stackpoole (1975) developed a simple procedure to predict the most suitable mixture of Actinomucor pusillus var. Lindt preparation and porcine pepsin for Cheddar cheese manufacture. It is based on their relative activities. The most acceptable cheeses were made with mixtures within 5% of the estimated required proportion of Actinomucor pusillus preparation. The mixtures of Actinomucor pusillus preparation and porcine pepsin produced more acceptable cheeses than either coagulant alone.

Role of Starter, Endogeneous Protease and Coagulant in Proteolysis of Cheese

Proteolysis in cheese is responsible for the transformation of the rubbery texture of green curd into the smooth bodied finish cheese (O'Keeffe, Fox and Daly, 1967). It also influences flavor and background flavor (Harper, 1959; Schormuller, 1968). The products of proteolysis have also been implicated to certain defects such as bitterness (Czulac, 1959; Lowrie and Lawrence, 1972; Tam and Whitaker, 1972; Visser, 1977a, c; Lowrie, 1977).

The four main proteolytic agents in ripened cheese are: milk coagulants, starter bacteria and their enzymes, non-starter bacteria and their enzymes, and endogeneous milk proteases. O'Keeffe et al. (1976) studied the contribution of coagulant and starter organisms to proteolysis in cheese. This was possible through the use of glucono delta lactone (GDL) or starter organisms as acidulants in cheeses manufactured under controlled bacteriological conditions. The similar electrophoretograms of aseptic GDL cheese and of starter cheese indicated that rennet was the major proteolytic factor in Cheddar cheese. Rennet also appeared to be responsible for the formation of large, medium, and small peptides detected by pH 4.6-soluble nitrogen and gel filtration techniques. Since only a limited range of free amino acids were produced in GDL cheeses, these were considered to be products of the action of microbial peptidases in Cheddar cheese.

Green and Foster (1974) also investigated the role of coagulant and starter bacteria in proteolysis of Cheddar cheese. In this study, cheese was prepared using either starter bacteria or GDL. To study the role of the starter, cheese was prepared with calf rennet and with porcine pepsin. This experiment was based on the observation of Green (1972) that all the porcine pepsin added as a coagulant in cheesemaking might be inactivated by the end of the cheesemaking process. They found, in contrast to

the work of O'Keeffe *et al*. (1976), that all cheesed made followed qualitatively similar courses of proteolysis, i.e., the bacterial proteinases in the cheese produced a pattern of protein breakdown similar to rennet.

In a more recent report (1978), O'Keeffe's group used porcine pepsin to yield a coagulant-free curd. They confirmed their previous findings and concluded that the coagulant is mainly responsible for the formation of large peptides. They suggested that the divergent findings of Green and Foster (1974) were due to their failure to produce a completely rennet-free cheese. Emmons, McGugan, Elliot and Morse (1962) had also investigated the effect of the strains of starter organisms on bitterness and protein breakdown in Cheddar cheese. The different strains gave no significant difference in acid-soluble nitrogen but showed significant differences in TCA-soluble nitrogen, and highly significant differences in amino-nitrogen. These findings agree with the works of O'Keeffe and coworkers (1976; 1978).

Visser (1977b) studied the protein breakdown in aseptically made Gouda cheese in which the action of rennet and of starter bacteria was selectively eliminated. Rennet appeared to be responsible for the development of most of the soluble nitrogen. Starter bacteria and milk proteases also made some contribution. In aseptic rennet-free and starter-free cheese a very small but measurable amount of

soluble nitrogen was detected. It was likely due to the endogeneous protease of milk. Gel filtration of soluble nitrogen compounds indicated that rennet liberated peptides of high and low molecular weight, and a very small amount of free amino acids. On the other hand, starter bacteria in cheese made without rennet produced low molecular weight peptides and free amino acids during ripening.

In normal cheese, with the enzyme systems acting together, rennet clearly stimulates the starter bacteria to produce amino acids and low molecular weight peptides. This is most likely due to the progressive degradation by the starter peptidases of the high molecular weight products of rennet action.

Visser and deGroot-Mostert (1977) continued studying the proteolysis in Gouda cheese using electrophoretic techniques. They found that in normal aseptic Gouda cheese α_{S1}-casein was degraded rapidly and the degradation was completed after one month of ripening. Beta-Casein was more resistant to proteolysis. After six months of ripening about 60% of β-casein was still intact. These findings are in agreement with those of Ledford, Chen, and Nath (1968). Those authors studied the proteolytic activity of commercial rennet on purified fractions of α_S- and β-casein and found that α_S-casein was less resistant than β-casein to rennet proteolysis.

From the experiments on starter-free cheese Visser et al. (1977) found that rennet was completely responsible for the degradation of α_{S1}-casein and for the decomposition of β-casein during the first month of ripening. From the experiments on rennet-free cheese they found that the starter bacteria could only degrade part of the α_{S1}-casein and part of the β-casein after several month of ripening. From the rennet and starter-free cheese it appeared that the milk protease was responsible for the formation of the minor caseins γ, R, TS and S during ripening of Gouda cheese. Its contribution to the breakdown of α_{S1}- and β-casein was low in comparison to that of rennet and starter bacteria.

Bitterness

The bitter flavor frequently observed when proteins are enzymatically hydrolyzed can be attributed to the accumulation of bitter tasting peptides. Casein appears to be extremely sensitive to bitter flavor development following proteolysis (Visser, 1977c). Therefore, the occurrence of the "bitter defect" in cheese can be considered dependent on the accumulation of bitter peptides that are produced by hydrolysis of the caseins. These peptides originate from both α_S- and β-casein (Mercier, Grosclaude, and Ribadeau-Dumas, 1972; Stadhouders and Hup, 1975; Visser, Slangen, and Hup, 1975). A bitter peptide

was isolated from whole casein and identified as the C-terminal end of β-casein. The sequence was Gly-Pro-Phe-Pro-Ile-Ile-Val-OH. Another bitter peptide was obtained from a rennin hydrolyzate of β-casein; it corresponds to segment 190-192 of β-casein (Mercier et al., 1972). Richardson and Creamer (1973) isolated a neutral peptide from bitter cheese and suggested that it probably was derived from the N terminal region of α_{S1}-casein.

Presently, there are two theories to explain the development of bitterness in cheese. In the first one (Czulac, 1959), rennet degrades casein to bitter peptides that accumulate in cheese unless the starter bacteria possesses peptidases that can degrade them. The more recent theory (Lawrence, Creamer, Gilles, and Martley, 1972; Lowrie and Lawrence, 1972; Lowrie, Lawrence, and Pearce, 1972) states that rennet is involved in the formation of predominantly non-bitter peptides of high molecular weight. These are converted to bitter peptides of low molecular weight by the enzymes of the starter organisms. Thereafter, the same starter organisms hydrolyze the bitter peptides to non-bitter peptides and amino acids.

To back this theory, Lowrie (1977) used milk infected deliberately with bacteriophage and found that this contamination prevented excessive development of bitter flavor. He concluded that since the enzymes of the starter streptococci give rise to bitter peptides, the reduction of the

starter population by massive phage attack in the last stages of cheese manufacture reduced the potential for formation of bitter peptides. The direct role of the starter bacteria in bitterness had already been proved by Lowrie, Lawrence and Peberdy (1974) who used high cooking temperatures to yield relatively low cell densities and reduced bitterness.

From results on work with aseptic Gouda cheese, Visser (1977a, c) concluded that the theory of Lowrie and coworkers for Cheddar cheese did not fit the pattern of bitterness development in Gouda cheese. Rennet alone could produce bitter peptides. Furthermore, an increase in the starter population was not sufficient to give a distinctly bitter cheese.

Sullivan, Mou, Rood and Jago (1973) reviewed the peptidase activities in both bitter and non-bitter strains with special reference to their action on bitter peptides. They concluded that all starter cultures have in their cytoplasm the aminopeptidases required for hydrolysis of bitter peptides to non-bitter products. The level of their activity is pH dependent, and at the pH of cheese ripening, i.e., at pH 5.0, the level of peptidase activity in the bitter strains is insufficient to hydrolyze the bitter peptides. These results may explain the failure of bitter strains to reduce the level of bitterness in cheese as reported in several works in the literature.

Holmes, Duersch and Ernstrom (1977) used a modified casein-agar diffusion test to measure the distribution of rennet activity between curd and whey. When calf rennet was used in cheese manufacture, approximately 35% of the rennet activity was destroyed prior to whey drainage; only 6% remained in the cheese following pressing. When cheese was made with microbial enzymes, there was no loss of activity during cheesemaking. However, most of the activity remained in the whey and only 2 to 3% was detected in the cheese after pressing. They suggested that since the percentage of active coagulant remaining in the finished Cheddar cheese was so small, especially in the case of the microbial enzymes, the role of the coagulant in flavor development should be minor, compared to that of the starter organism.

Stadhouders and Hup (1975) studied the factors affecting bitter flavor in Gouda cheese. They found that when the initial pH of the cheese milk was low more rennet was retained in the cheese. Also, when high temperatures were used during cooking less rennet was retained in the cheese and there was a decrease in the starter population. These factors were associated with less intensity of bitter flavor in the cheese. Salt was also found to reduce bitterness. Its effect was not clearly understood but it could be related to the hydrolysis of β-casein and subsequent formation of bitter peptides previously mentioned.

A number of other works support either of the two theories. Therefore it is safer to conclude that the bitter flavor in cheese is a complex defect and it seems to be affected by many factors. In general, it results from a disproportion between the formation and the degradation of bitter peptides resulting in their accumulation above the threshold value for bitterness detection in cheese.

Bitter peptides have certain characteristic properties of which hydrophobicity and the nature of the terminal residues (particularly the C-terminal residue) appear to be the most important (Matoba and Hata, 1972; Sullivan and Jago, 1972). Visser et al. (1975) isolated bitter peptides from whole and β-casein which showed a high content of hydrophobic amino acid residues. The same observation was made by Harwalkar (1972) when he isolated an astringent fraction from Cheddar cheese, and by Visser (1977c) from Gouda cheese.

In contrast, Pelissier and Manchon (1976) compared the ability of various proteases to produce bitter hydrolyzates from cow's, goat's and ewe's caseins. When the amount of α_{S1}-casein was small, less bitterness developed. Different results were observed from one protease to the other. Therefore they concluded that the total hydrophobicity of a protein may not be the only factor determining bitterness, since β-casein is more hydrophobic than α_{S1}-casein. Secondly, the nature of the protease used has a significant

influence on the development of bitterness.

Identification of Milk Coagulants in Cheese Making

The U.S. Customs regulations define rennet as a derivative from animal stomachs (Prager, 1977) and it can be imported free of duty. On the other hand, fermentation derived coagulants are subject to duty when imported. This and other reasons call for standardized procedures for identification of rennet and rennet substitutes.

DeKoning (1974) has surveyed different methods for the characterization of the different enzymes. He considers the technique of isoelectric focusing in combination with immunological methods as the most selective identification procedure.

A common method used is the casein-agar diffusion technique developed by Cheeseman (1963) and modified by Lawrence and Sanderson (1969). Here, zones of precipitation form in a thin layer of agar containing calcium caseinate when proteolytic enzymes diffuse through the agar. The number and width of the precipitation zones formed depends on the enzyme used.

The use of polyacrylamide gel electrophoresis for identification of the coagulant used in cheese manufacture was attempted by Edwards and Kosikowski (1969). Wide differences on the electrophoretic pattern of Cheddar cheese were observed depending on the type of milk coagulant

used. However, it was very difficult to accurately identify a coagulant by this procedure because of the intermixing of α_S- and β-casein components. When Prager (1977) compared the electrophoretic patterns of aqueous solutions of the coagulants a characteristic pattern could be seen for each type of enzyme. Another polyacrylamide gel electrophoresis system was developed by Shovers, Fossum, and Neal (1972) for qualitative evaluation of the coagulants. In this procedure, after the electrophoretic run, visualization of the zones is achieved by layering the gel-slab with milk. The areas that have milk coagulating activity will exhibit zones of clotted milk that can be easily seen. These zones have different patterns depending on the coagulant used.

Aside from the qualitative identification of the different milk coagulants, it is imperative to be able to quantitate the composition and activity of the clotting agents. This may help in determining their suitability for cheese manufacture.

O'Leary and Fox (1974) described a procedure for the quantitative determination of calf rennet, bovine pepsin, porcine pepsin and proteases from Actinomucor miehei, and Actinomucor pusillus. The method uses chromatography on DEAE cellulose in piperazine buffer to fractionate the enzymes. This step is followed by selective pH-temperature denaturation. Collin, Martin, Garnot, Ribadeau-Dumas, and

Mocquot (1981) and Garnot, Thapon, Mathieu, Maubois, and Ribadeau-Dumas (1972) separated chymosin and bovine pepsin A from commercial rennet extract also by DEAE cellulose chromatography. The activity of the individual fractions was assayed by measuring the clotting time on Berridge's substrate standardized with pure chymosin and pure bovine pepsin A.

DiGregorio, Sisto, and Morisi (1979) found that sepharose 4B quinonated and deactivated by an amine is a biospecific sorbent for chymosin. This selective absorption was used for developing a method for both purification and routine analysis of commercial rennets. The test involves a chromatographic elution of the sample on the quinonated sepharose column and milk clotting activity measurements. It seems simple, inexpensive and has the particular feature of operating directly in crude commercial samples.

Another quantitative procedure is based on the stability of porcine and bovine pepsin at pH 2.0-2.5 in 5.0 M urea, and the complete denaturation of chymosin, Actinomucor miehei and Actinomucor pusillus proteases under the same conditions (Mulvihill and Fox, 1977a).

Rothe, Harboe and Martiny (1977) successfully used rocket immunoelectrophoresis using monospecific antibody preparations against rennin, bovine pepsin A and bovine pepsin B. Another immunological diffusion procedure was

developed by Matheson (1981). It takes advantage of both the sensitivity of the casein agar diffusion method and of the selectivity of a specific rennin antiserum, thereby allowing the activity of rennin to be determined in cheese in the presence of other milk coagulating enzymes. More recently, Collin, De Reta, and Martin (1982) prepared rabbit monospecific antisera from the six most widely used milk clotting enzymes, i.e., rennin, bovine pepsin, porcine pepsin, and proteases from Actinomucor miehei, Actinomucor pusillus and Endothia parasitica. These antisera were used to identify enzymes added to calf or bovine rennets by a double radial immunodiffusion technique.

Residual Coagulants in Whey

Because they are thermosensitive, the heat stability of the coagulant was not considered a problem when only rennet or pepsin were used. The new microbial coagulants, however, are more heat stable. Therefore, prior to concentration and drying, the whey requires a more severe heat treatment.

Thunell et al. (1979) investigated the conditions necessary for thermal inactivation of these enzymes. They found that the Actinomucor miehei protease was the most heat stable coagulant at all pH values followed by Actinomucor pusillus protease, calf rennet, bovine pepsin, Endothia parasitica protease and porcine pepsin. The heat

stability of all the enzymes increased with decreasing pH except for Endothia parasitica. Hyslop, Swanson and Lund (1979) reached the same conclusions. They found that for 90% inactivation in 15 seconds and at pH 6.0, heat treatments of 77 C, 71 C and 67 C are required for Actinomucor miehei protease, Actinomucor pusillus protease, and rennet-pepsin mixture, respectively. Because of this high thermostability a number of thermosensitive enzymes from microbial origin have been developed. Ramet and Weber (1981a, b) have investigated the technological properties of a new thermosensitive milk clotting enzyme from Actinomucor miehei. The production, processing, yield and organoleptic properties of Camembert and Saint Paulin cheeses appear unchanged by the new coagulant.

New Approaches in Milk Coagulation

As seen in the course of this review, the most used alternative for overcoming the shortage of calf rennet is its replacement by a coagulant of similar properties. A series of novel approaches have been recently reported, however.

The use of renneted dry milk in milk coagulation was considered (Aftan, Bennasar, Tarodo, and Fuente, 1981). The powder was obtained by atomization of milk powder after rennet action at low temperatures; simple heating after reconstitution caused coagulation. This powder can be

added as a coagulating agent to fluid milk in a specific amount so that the amount of whey drained can be controlled.

Knowing that enzymes can be used more economically and efficiently if they are immobilized, Cheryan, VanWyk, Richardson and Olson (1976) and Taylor, Cheryan, Richardson and Olson (1977) used pepsin covalently attached to porous glass or to inorganic supports. The milk was acidified with phosphoric acid to pH 5.6-5.9 at temperatures below 15 C to prevent coagulation. The authors reported problems of reduction of flow rate due to accumulation of curd particles and some inactivation of the immobilized enzyme at the pH of milk. Recent reports by Ohmiya, Tanimura, Kobayashi and Shimizu (1978, 1979) claim the successful immobilization of an alkaline protease from Bacillus subtilis on an anion exchange resin by glutaraldehyde. By reversal of the ripening of the milk and the renneting step during cheese manufacture, and by raising the pH of milk to 7.0, coagulation of the milk in the column was prevented. Coagulation was subsequently induced by lowering the pH to 6.2 by the lactic acid fermentation.

Shindo, Sakurada, Niki and Arima (1980) immobilized chymosin using paraffin wax and produced Gouda cheese that was compared with conventionally made Gouda type cheese. Comparisons were based on electrophoretic patterns of degradation and free amino acid analyses. It was suggested that immobilized chymosin would be useful for cheesemaking.

Recently, DiGregorio and Sisto (1981) studied the potential use of partly esterified proteins as coagulating agents. Since the coagulation process results from the reduction of the negative charge of the casein micelles by rennet, a cationic polyelectrolyte could come into contact with the casein micelles in milk and interact with their negative charge thus allowing coagulation by a binding-flocculation mechanism.

Another means by which the amount of milk coagulating enzyme can be reduced is based on the fact that most of the coagulating enzyme goes to the whey and it is not recovered in cheese. By using ultrafiltration devices Chojnowski, Poznanski, Jedrychowski, and Reps (1981) have studied the possibility of reusing the enzymes recovered from whey. Tilsit cheese was manufactured from milk enriched with 10% whey concentrate and in this way the amount of rennet required to produce sufficient firmness of the curd was reduced to 2/3 the normal amount.

Finally, the advent of Recombinant DNA technology and the current surge of interest in biotechnology opens the possibility of a more economically viable commercial production of rennin.

MATERIALS AND METHODS

Chemicals and Materials

The principal chemicals used in this study along with their source are listed in the Appendix, Table A1. All chemicals used were reagent grade. Distilled, deionized water was used in the preparation of all the solutions.

The cheddar cheese was made from whole milk obtained from Holstein cows of the Michigan State University dairy herd.

Equipment

Equipment and instrumentation specific for a certain experiment will be referred to in the appropriate section.

Experimental Procedure

Manufacture of American Cheddar-type Cheese

The milk used for the manufacture of the experimental cheese ranged in total solids content from 11.34% to 12.01%, in fat content from 3.12% to 3.41% and in protein content from 3.40% to 3.55%.

Each week about 600 kg lots of milk were collected immediately after milking and submitted to pasteurization at 62.7 C for 30 min. After cooling to 32 C batches of

100 kg each were placed in three 50 gal stainless steel cheese vats for immediate use. The other 300 kg were stored at 2-3 C for use after 48 h.

The availability of the equipment made it impossible to make more than 6 lots of cheese per week. Therefore in 4 weeks a total of twenty-four 97.71 ± 1.15 kg lots of raw whole milk were used. Six different experimental treatments were applied. To prevent uncontrolled sources of variation (changes in milk composition, storage of milk, etc.) from influencing the responses in a systematic manner, the different treatments were chosen randomly and every treatment was applied to either non-stored or stored milk twice.

The manufacturing procedure was an adaptation of the methods given by Wilster (1974) and Kosikowski (1978). After placing the pasteurized milk in the 50 gal stainless steel cheese vats the temperature was adjusted to 31.1 C by circulating cold water or steam in the vat jacket. One percent of a DVS Redi-Set, frozen lactic culture #980 containing four strains of Streptococcus cremoris (Chr. Hansen's Laboratory, Inc., Milwaukee, Wisconsin) was added to the milk. Immediately after the starter 3.22 ml of double strength Annatto color (Marshall Division, Miles Laboratories, Elkhart, Indiana) was added to give the cheese a light orange color. When the titratable acidity of the milk had gone up about 0.02% it was considered

ripened. This ripening period ranged from 1 h to 1 h 10 min.

Since the purpose of this study was to compare various coagulants and their role in casein breakdown, the next step, i.e., the addition of coagulant, was the only variation from lot to lot.

Six coagulating enzymes were used:

1. Rennet extract, single strength, prepared by extraction of the 4th stomach of the suckling calf (Marschall Division, Miles Laboratories, Elkhart, Indiana)

2. Marzyme II, single strength, produced by the pure culture fermentation of the organism Actinomucor miehei (Marschall Division, Miles Laboratories, Elkhart, Indiana)

3. Bovin Rennet, single strength. Extract produced from adult bovine animals. Chr. Hansen's Laboratory Inc., Milwaukee, Wisconsin)

4. Emporase, single strength. Microbial rennet produced from controlled pure culture fermentation by Actinomucor pusillus Lindt (Dairyland Food Laboratories, Inc., Waukesha, Wisconsin)

5. Beemase, single strength. A blend of microbial rennet produced from Actinomucor pusillus Lindt and bovine rennet (Dairyland Food Laboratories, Waukesha, Wisconsin)

6. Econozyme, double strength. A blend of calf rennet and porcine pepsin (Pfizer Inc., Milwaukee, Wisconsin)

The amount of coagulant used was that recommended by the manufacturer. It was diluted in 30 times its volume of cold water and poured in an even stream around the vat. The milk was then stirred for 2 to 3 min and left quiescent until it curdled. The curd was ready to cut in about 45 min. It was cut with 1 cm horizontal and vertical wire knives. Within 5 min after cutting, the curds were gently stirred for about 10 min to prevent matting. The acidity of the whey immediately after cutting ranged from 0.095% to 0.105%.

Next was the cooking process. The temperature was increased at a rate of 1 F every 5 min up to 100 F (37.8 C). The average cooking time was 1 h 36 min.

To determine if the curd was cooked and had the proper firmness any of the following criteria was used:

a. curd contracts to less than half of its original size. When a handfull is pressed together, the pieces should fall apart with no tendency to stick together.

b. the curd should feel hard with no soft or mushy center.

c. whey acidity should be 0.02% higher than that at the moment of cutting.

When the curd was considered ready, the whey was quickly drained and the curd stirred.

The following step consisted of piling the curd for matting. In about 10 to 15 min the matted curd was cut

into slabs and the cheddaring process began. The purpose of cheddaring was to produce cheese with a close knit texture and a waxy body with good slicing property. The slabs were kept warm (37.8 C) and turned every 10 to 15 min to facilitate draining of the whey. Cheddaring required an average of 67 min. When the titratable acidity of the whey was about 0.45 to 0.55 percent and the pH of the slabs was approximately 5.1 to 5.2 the slabs were ready for milling. At this point the curd had a smooth appearance, similar to roasted chicken's breast.

After milling, the curd was forked until its temperature dropped to about 32 C. Salt was then added (1.5% of the cheese) and mixed for about 15 min to obtain homogeneous distribution in the cheese curds. Wilson square stainless steel hoops (9.07 kg) were then filled with the cheese curd and pressed at 30 psi (2.10 kg/cm^2) overnight at room temperature (23 C). The next morning the cheese blocks were taken out of the hoops and vacuum packed in Cryovac Barrier bags. The cheese blocks were kept at 8.9 C and 85% humidity in the curing room and ripened up to 9 months.

A copy of the production-sheet used during the manufacture of the Cheddar cheese is shown in Figure 1.

Preparation of Whole Casein

The whole casein to be used as a standard for comparison in the electrophoretic studies was prepared according

Cheddar Cheesemaking Record:

Date_____ 19__

Operator:

_____

Quantity of Milk:

_____

Fat Content:

_____

Quantity of Cheese:

_____

Yield:

_____

Process → / Results ↓	Stock Milk	Pasteurization	Lactic Starter	Color	Setting Milk	Rennet	Coagulation Time	Cutting	Stirring	Cooking	Draining Whey	Cheddaring	1st Turning Over	2nd Turning Over	3rd Turning Over	Milling	Salting	Pressing	Curing
Titratable Acidity																			
pH																			
Temperature																			
Time																			
Duration																			
Quantity																			

Observations:

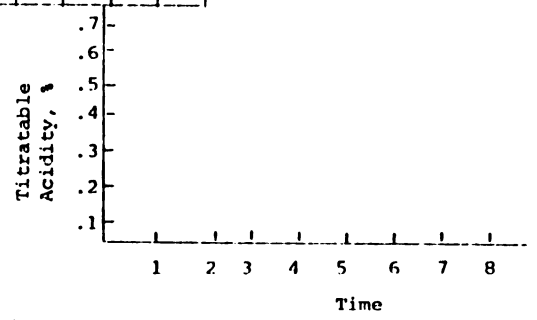


Figure 1. Production sheet for the manufacture of American Cheddar-type cheese.

to a method described by Vanderpoorten and Weckx (1972). Whole milk was obtained from Holstein cows of the Michigan State University dairy herd; the fat was removed by centrifugation for 30 min at 754 xg. Final fat content was 0.1% or less. The skimmilk obtained in this way was slowly acidified to pH 4.6 with 1 N HCl. The precipitate was washed three times with distilled water and extracted 5 h at pH 4.0 in acetic acid so that the milk proteases were removed. The whole casein obtained was washed 3 more times with distilled water, lyophilized and stored at 0 C.

Analytical Procedure

Whole milk was analyzed for total solids, total nitrogen and fat before using. Whey was analyzed for total solids, total nitrogen and fat.

Cheese curds were analyzed for residual coagulant after cooking. The cheese samples were analyzed immediately (0 time) for moisture, fat, salt, ash, pH, total nitrogen, pH 4.6-soluble nitrogen, 12% TCA-soluble nitrogen, and residual coagulant. Residual casein fractions were also determined by discontinuous polyacrylamide gel electrophoresis (DISC-PAGE) and by sodium dodecyl sulphate discontinuous polyacrylamide gel electrophoresis (SDS-DISC-PAGE).

After 1, 3, 6 and 9 months of ripening the cheese samples were analyzed for moisture, pH, pH 4.6-soluble nitrogen,

12% TCA-soluble nitrogen and residual coagulant. Residual casein fractions were also determined by DISC-PAGE. After 3, 6 and 9 months of ripening the residual casein fractions were determined by SDS-DISC-PAGE.

Milk clotting and proteolytic activities of the enzyme preparations employed and their electrophoretic patterns were also determined.

Except for the samples used for moisture determination, all cheese, milk and whey samples were sealed in barrier bags (6 oz Whirlpak bags) and held frozen at -29 C until the time of actual analysis.

Compositional Analysis

Total Solids

Total solids in milk, whey and cheese were determined by a vacuum oven method (AOAC, 1975) as follows:

1. Two grams of a sample were accurately weighed into a preheated and preweighed round flat-bottom metal dish.

2. The sample was partially dried on a Corning hot plate until the first traces of brown color appeared.

3. The dish was then placed in a vacuum oven (Precision-Thelco oven model 19) and kept at 100 C for 4 h and under pressure of less than 100 mm Hg.

4. The dish was removed from the oven, cooled, and weighed.

5. The percentage moisture could be calculated from the differences in weight of the original samples and the dried solids residue.

For each sample the test was done in duplicate.

Fat

The fat content of the milk, whey and cheese samples was determined by the Roese-Gottlieb method with Mojonnier modification (Milk Industry Foundation, 1959). Steps followed were those described by Mojonnier Bros. Co. (1925) for determination of fat in milk, whey and cheese.

First Extraction

1. About 10 g of milk, 10 g of whey or 1 g of shredded cheese were accurately weighed into extraction flask using a 10-g pipette or a butter boat.

2. Only in the case of the cheese sample, 8.0 ml of distilled water were added to the extraction flask and mixed thoroughly.

3. Then 1.5 ml of ammonium hydroxide were added and mixed thoroughly.

4. Ten milliliters of 95% alcohol were added and the bottle was shaken for 30 sec.

5. Twenty five milliliters of ethyl ether were added, and the bottles were shaken vigorously for 20 sec.

6. Twenty five milliliters of petroleum ether were added and, again, the bottles were shaken vigorously for 20 sec.

7. The extraction flasks were centrifuged at 60 rpm for 30 sec.

8. The ether mixture containing the extracted fat was poured off into previously weighed fat dishes. Prior to use, the empty fat dishes had been heated in vacuum oven at 135 C for 5 min and cooled in desiccator for 7 min.

Second Extraction

1. Now, neither distilled water nor ammonium hydroxide was used.

2. Five milliliters of alcohol were added to the residue in the flask and it was shaken for 20 sec.

3. Then 15 ml of ethyl ether were added and the bottle was shaken for 20 sec.

4. Fifteen milliliters of petroleum ether were also added and the bottle was shaken for 20 sec.

5. The extraction flasks were centrifuged at 60 rpm for 30 sec.

6. The ether-fat solution was poured into the same fat dish used in the first extraction. When it was necessary to raise the dividing line between the fat soluble and water soluble fractions in the extraction flask, a small amount of distilled water was added. Care was taken not to pour any of the water soluble fraction into the dish.

7. The fat dishes were placed in an electric hot plate until the ether was evaporated; then, they were transferred to an oven and kept 5 min at 135 C.

8. The dishes were finally cooled for 7 min in the desiccator and weighed. For each sample the test was done in duplicate.

Salt

Determination of salt in cheese samples was performed by the modified Volhard test described by Kosikowski (1978) and with some modifications:

1. Three grams of shredded cheese were weighed and quantitatively transferred to a 300 ml Erlenmeyer flask.

2. Twenty five milliliters of 0.1 N $AgNO_3$, 10 ml of halogen-free HNO_3, and 50 ml of distilled water were added.

3. The mixture in the flask was heated to boiling under a hood.

4. As the mixture boiled, 15 ml of fresh saturated $KMNO_4$ solution were added, followed by two 5 ml portions of the same oxidant solution. Each time, the $KMNO_4$ was added after the purple color of the mixture had changed to yellow.

5. The digested yellow mixture was then cooled to room temperature and 2 ml of acetone and a few drops of saturated ferric ammonium sulfate indicator were added.

6. The contents were titrated directly with 0.1 N KSCN to a brick-red end point.

7. Calculations were made using the formula:

$$\frac{(25 \text{ ml } AgNO_3 \times \text{Normality} - \text{ml KSCN} \times \text{Normality}) \times 0.058 \times 100}{\text{sample wt., g}} =$$

% NaCl

For each sample the test was done in duplicate.

Ash

Ash in cheese samples was determined by the method described by the Association of Official Analytical Chemists (AOAC, 1975) with some modifications.

First, approximately 2 g of sample were accurately weighed into a preweighed round flat-bottom metal dish of 5 cm diameter. The dish with the sample was placed in the vacuum oven at 100 C (±0.1 C) and pressure of less than 100 mm of Hg for 4 h to dry to a constant weight.

At the end of drying the dish was removed from the oven and cooled. Approximately 0.5 g of dry matter was weighed into a preweighed crucible. This crucible was placed on a Corning hot plate to ignite the dry matter and avoid spattering afterwards. When flame ceased, ignition was completed in a Sybron-Thermolyne muffle furnace (Model 10500) at 550 C for 20 h. The crucible containing the grey-white residue was removed from the

furnace into a desiccator. After cooling to room temperature the crucible was weighed to determine percentage of ash. The test was done in duplicate.

Total Nitrogen

The total nitrogen in milk, whey and cheese was determined by the Micro-Kjeldahl method for determination of total nitrogen (AOAC, 1975):

1. The sample size used was that which would require 3-10 ml 0.02 N HCl for titration; it was accurately weighed in a 30 ml Kjeldahl digestion flask.

2. 1.9 g of K_2SO_4, 40 mg of HgO and 3-3.5 ml concentrated H_2SO_4 were added to the digestion flask along with boiling chips.

3. The samples were digested for 90 min in a digestion rack heater under a hood.

4. After digestion, the flasks were cooled and about 3 ml distilled water were added to dissolve the solids.

5. The digest was then transferred to the distillation apparatus and the flask was rinsed a few times with distilled water.

6. Ten milliliters of a sodium hydroxide-sodium thiosulphate solution (60 g NaOH and 5 g $Na_2S_2O_3$ x 5 H_2O to 100 ml distilled water) were added to the digest in the distillation apparatus.

7. A 50 ml Erlenmeyer flask containing 5 ml saturated H_3BO_3 solution and 4 drops of methyl red-methylene blue

indicator was placed under a condenser with tip extending below the surface of the solution.

8. Approximately 20 ml distillate were collected and titrated to end point with 0.02 N HCl solution.

9. A blank was run simultaneously. The formula used for the calculations was:

$$\frac{(\text{ml HCl sample} - \text{ml HCl blank}) \times \text{normality} \times 14.007 \times 100}{\text{sample wt., mg}} = \% \text{ nitrogen}$$

For each sample the test was done in duplicate.

Monitoring of Cheese Ripening

pH

pH measurements were made with a Chemtrix type 60A digital pH/mv meter equipped with an Orion (model 91-63) pH electrode designed for surface measurements. Before testing the pH meter was standardized with standard buffer solutions of pH 4.01 and pH 7.0; then, it was manually set to the temperature of the product. pH was determined in all samples to the nearest 0.01 pH unit. After the first determination, the electrode was moved to test two other areas of the same cheese. An average of the three pH readings is reported in the Results section.

pH 4.6-Soluble Nitrogen

Determination of soluble nitrogen in cheese samples was done following a technique based on the method of Sharp

(Kosikowski, 1978) with some modifications. The extraction solutions needed were:

A. Stock solution:

57.5 ml glacial acetic acid

136.1 g sodium acetate ($3H_2O$)

47.0 g sodium chloride

8.9 g calcium chloride (anh)

Add distilled water to make volume to 1000 ml

B. Extraction solution:

Dilute 250 ml stock solution to 1000 ml with distilled water.

The soluble nitrogen was extracted as follows:

(1) In a 6 oz. Whirlpak bag 1.5 g of cheese were accurately weighed. A small amount of extraction solution at 50 C was added and the suspension was mixed thoroughly.

(2) This mixture was quantitatively transferred to a 25 ml volumetric flask and volume was made to 25 ml with more extraction solution.

(3) The flask was placed in a water bath at 50 C and maintained at this temperature for 1 h.

(4) The suspension was then filtered through a Whatman No. 1 fluted filter paper and 3 ml of the filtrate were placed in a 30 ml Micro-Kjeldahl flask.

(5) Digestion and distillation steps were conducted as for total nitrogen. For each sample the test was done in duplicate.

12% TCA-Soluble Nitrogen

12%-TCA soluble nitrogen in cheese was determined following the procedure described by O'Keeffe et al. (1976) and Reville and Fox (1978) with some modifications.

(1) Two to three grams of cheese were accurately weighed into a 6 oz. Whirlpak bag. A small amount of water at 75 C was added and the suspension was mixed thoroughly.

(2) The mixture was quantitatively transferred to a 25 ml volumetric flask and volume was made to 25 ml with more distilled water.

(3) The flask was placed in a water bath at 75 C and maintained at this temperature for 5 min.

(4) The suspension was cooled and filtered through a Whatman No. 1 fluted filter paper.

(5) To a 10 ml aliquot 15 ml of 20% TCA-solution were added so that the final 25 ml would have 12% TCA.

(6) A precipitate was immediately formed and it was removed by centrifugation (Sorvall SuperSpeed centrifuge model RC2-B) at 20,000 xg for 30 min at 2 C. The supernatant (5.0-7.0 ml) was placed in a 20 ml Micro-Kjeldahl flask.

(7) Digestion and distillation steps were conducted as for total nitrogen. For each sample the test was done in duplicate.

Discontinuous Polyacrylamide Gel Electrophoresis (DISC-PAGE)

DISC-PAGE was performed according to the method of Orstein (1964) and Davis (1964) with modifications by Malachouris (1969) for whey proteins, casein and clotting enzymes and adapted for cheese samples.

Electrophoresis was conducted in glass tubes with a length of 12.5 cm and an inner diameter of 6 mm. The tubes were cleaned prior to use by washing with soap and water, soaking in chromic acid, rinsing with distilled water, and soaking in Photo-flow (1:200).

Electrophoresis Solutions

The stock solutions needed were:

(1) Running gel buffer, pH 8.0, 0.380 M Tris HCL was prepared by dissolving 46.018 g of Tris in about 950 ml distilled water; 420 g of urea were added to make the buffer 7 M. The pH was adjusted to 8.9 with concentrated HCl and the volume was made to 1000 ml with distilled water.

(2) Stacking gel buffer, pH 6.7, 0.062 M Tris-HCl was prepared by dissolving 7.508 g of Tris in about 950 ml distilled water; 420 g of urea were added to make the buffer 7 M. The pH was adjusted to 6.7 with concentrated HCl and the volume was made to 1000 ml with distilled water.

(3) Electrode buffer, pH 8.3, 0.046 M Tris-glycine was prepared by dissolving 5.57 g of Tris in about 700 ml

distilled water. The pH was adjusted to 8.3 with 2 M glycine solution and the volume was made to 1000 ml with distilled water.

(4) Running gel solution, 25% (w/v) acrylamide solution was prepared by dissolving 24.83 g of acrylamide monomer and 0.64 g of Bisacrylamide in 75 ml of the running gel buffer and making it to 100 ml with the same buffer. This gave a stock solution with 25% total acrylamide and 2.56% crosslinker (bisacrylamide).

(5) Stacking gel solution, 6.25% (w/v) acrylamide solution was prepared by dissolving 5 g of acrylamide monomer and 1.25 g of bisacrylamide in 75 ml of the stacking gel buffer and making it to 100 ml with the same buffer.

(6) Ammonium persulfate solution, 5% (w/v) solution made in 7 M urea.

(7) N,N,N',N'-tetramethylethylene diamine (TEMED).

(8) Extraction solution was 7 M urea.

(9) Bromophenol blue, 1% (w/v) solution made in stacking gel buffer.

(10) Saturated sucrose solution made in stacking gel buffer.

(11) Staining solution was prepared with 25% (v/v) isopropanol, 10% (w/v) acetic acid and 0.05% (w/v) coomassie brilliant blue R-250 in distilled water.

(12) Destaining solution was prepared with 4% (v/v) acetic acid and 10% (v/v) isopropanol in distilled water.

All solutions were filtered before using.

Gel Preparation

(1) The dry tubes were marked with a felt-tip pen at distances 10.0 and 11.6 cm from the bottom.

(2) The bottom of each tube was fitted with a small square of plastic wrap held on with a rubber adapter. Each tube was then placed in the leveled rack.

(3) A gel solution of the desired concentration (9%) was prepared by combining the appropriate volumes of running gel solution and running gel buffer to give a final volume of 25.0 ml.

(4) To this gel solution 20 µl of Temed and 0.3 ml of ammonium persulfate were added.

(5) The gel solution was then transferred to the glass tubes with a syringe and an 18 gauge needle. Each tube was filled to the 10.0 cm mark, overlayed with water, and allowed to polymerize overnight.

(6) The stacking gel was prepared by mixing 5.0 ml of the stacking gel solution with 1 g of sucrose. The volume of the solution was made to 10.0 ml with stacking gel buffer; 40 µl of ammonium persulfate and 10 µl of Temed were added.

(7) After polymerization of the running gel, the water layer was removed and the stacking gel layered on

top of the running gel. Each tube was filled to the 11.6 cm mark, overlayed with water, and allowed to polymerize for one h.

Sample Preparation

Cheese samples for electrophoretic analyses were prepared following a procedure reported by Ledford et al. (1966), with some modifications; 0.05 g of cheese was dissolved in 0.8 ml of distilled water and 2.0 ml of 7 M urea. The sample solution was then warmed to 37 C for 1 h to effect a layering of fat thus facilitating removal of the aqueous phase for electrophoresis. To demonstrate κ-casein 4 drops of mercaptoethanol were added 45 min prior to electrophoresis. Right before the electrophoretic run, 3 µl of Bromophenol blue and 100 µl of saturated sucrose solution were added. A sample containing approximately 45 µg of protein was added to each tube.

Whole casein was used as a standard for comparison with the cheese samples. A casein solution (0.5% w/v) was prepared with the stacking gel buffer containing the 7 M urea. To 1 ml of sample 3 µl of bromophenol blue and 100 µl of saturated sucrose solution were added prior to the electrophoretic run. A sample containing approximately 45 µg of protein was added to each tube.

Electrophoretic Conditions

Electrophoresis was carried out in a water-cooled Bio-Rad Model 150A electrophoresis apparatus and power was

supplied by a Bio-Rad Laboratories Model 500 power supply. Destaining was performed in a Bio-Rad Laboratories Model 1200A electrophoretic destainer.

(1) The glass tubes containing the gels were transferred to the electrophoretic apparatus. The anodic and cathodic buffer reservoirs were filled with the electrode buffer. The protein samples were then carefully layered on top of the stacking gel in volumes of 10-20 µl.

(2) Electrophoresis was initially conducted at 1 mA/tube until the samples entered the running gel. After that a constant current of 2 mA/tube for the cheese and casein samples was maintained. Electrophoresis was concluded when the marker dye had migrated to the bottom of the gels.

(3) The gels were removed from the glass tubes and stained in 0.05% (w/v) coomassie brilliant blue R-250 overnight.

(4) Electrophoretic destaining was performed for 75 min in the destaining solution.

Gel Densitometry

Gels were scanned using a Beckman DU Spectrophotometer, Model 2400 equipped with a gel scanner Model 2520 and a photometer 252 by Gilford Instrument Laboratories, Inc.

This system was interfaced to a Hewlett-Packard integrator Model 3380S. The gels were scanned at a rate of

0.5 cm/min and a chart speed of 1 cm/min. Start delay and slope sensitivity settings were 0 and 3.0 mV/min, respectively.

DISC-PAGE gels were scanned at a wavelength of 550 nm and attenuation was set at 64. The relative areas of the individual proteins were recorded. The relative mobility of the bands was assessed from the total length of the gels and from the distances migrated by individual proteins.

Sodium Dodecyl Sulfate-Discontinuous Gel Electrophoresis (SDS-DISC-PAGE)

The improved method of discontinuous electrophoresis in acrylamide gels containing SDS (Laemmli, 1970; King and Laemmli, 1971) was used with some modifications.

20x20 cm glass plates of identical thickness and spacer pieces were used. They were detergent washed, distilled water rinsed and dried before use.

Electrophoresis Solutions

The stock solutions needed were:

(1) Stacking gel buffer 0.5 M Tris-HCl pH 6.8 with 0.4% SDS was prepared by dissolving 30.3 g Tris and 20 ml of 10% sodium dodecyl sulfate aqueous solution in about 400 ml of distilled water. The pH was adjusted to 6.8 with concentrated HCl and the volume was made to 500 ml with distilled water.

(2) Running gel buffer. 1.5 M Tris-HCl pH 8.8 with 0.4% SDS was prepared by dissolving 90.9 g Tris and 20 ml of 10% sodium dodecyl sulfate aqueous solution in about 400 ml of distilled water. The pH was adjusted to 8.8 with concentrated HCl and the volume was made to 500 ml with distilled water.

(3) Electrode buffer. Tris glycine buffer pH 8.5 with 0.1% SDS was prepared by dissolving 6 g of Tris, 28.8 g of glycine, 10 ml of 10% sodium dodecyl sulfate aqueous solution, and 10 ml 0.2 M sodium EDTA in distilled water to a volume of 1000 ml.

(4) Sample buffer was prepared by dissolving 6.06 g Tris, 42 g urea, 20 g SDS and 5 ml of β-mercaptoethanol in distilled water to a volume of 100 ml.

(5) Acrylamide stock solution. 20% acrylamide, 0.49% bisacrylamide was prepared by dissolving 20 g acrylamide monomer and 0.49 g bisacrylamide in 100 ml distilled water.

(6) Ammonium persulfate solution. 2% (w/v) aqueous solution.

(7) N,N,N',N'-tetramethylene diamine (Temed).

(8) Bromphenol blue. 1% (w/v) solution.

(9) Saturated sucrose solution.

(10) Fixing solution containing 10% ethanol and 7.5% glacial acetic acid.

(11) Staining solution was prepared by dissolving 1.6 g coomassie brilliant blue G-250, 40 ml ethanol, and 120 ml

of 70% perchloric acid in distilled water to a volume of 4000 ml.

(12) Destaining solution was prepared with 7.5% glacial acetic acid in distilled water.

(13) Final solution containing 10% ethanol and 2% glycerol. All solutions were filtered before use.

Slab-gel Preparation

(1) A gel solution of the desired concentration (13%) was prepared by combining 19.5 ml of the 20% acrylamide stock solution, 2.4 ml of distilled water, 7.5 ml running gel buffer, 0.6 ml of ammonium persulfate, and 15 µl of Temed. This gave a final volume of 30 ml.

(2) This running gel solution was poured between the clamped plates until the space was filled. The top spacer piece was then inserted carefully so that no air bubble was formed. The gel was allowed to polymerize overnight.

(3) The next day the top spacer piece was removed and the area of the plate above the gel was washed.

(4) The stacking gel solution containing 5% acrylamide was prepared by mixing 5 ml of the 20% acrylamide stock solution, 5 ml of the stacking gel buffer, 15 µl Temed, 0.25 ml ammonium persulfate solution, and 9.85 ml water.

(5) The stacking gel solution was poured on top of the running gel filling the spaces between the clamped plates. A toothed top spacer piece was inserted leaving no bubbles beneath it. The gel was allowed to polymerize for 1 h.

Sample Preparation

Cheese samples for electrophoretic analyses were prepared by dissolving 0.05 g of cheese on 3 ml of the sample buffer, and adding 3 µl of Bromophenol blue and 100 µl of saturated sucrose solution. The sample solution was left at room temperature for 1 h. A sample containing approximately 60 µg of protein was added to each well in the slab.

Whole casein was used as a standard for comparison with the cheese sample. A casein solution (0.5% w/v) was prepared with the sample buffer and to 1 ml of it, 3 µl of bromphenol blue, and 100 µl of saturated sucrose solution were added. A sample containing approximately 70 µg of protein was added to a well in the slab.

For determination of molecular weights, a low molecular weight calibration kit (Pharmacia Fine Chemicals, Inc.) was used. The protein mixture contained in the kit consisted of phosphorylase b (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and α-lactalbumin (14,400). 100 ml of a buffer containing 1% SDS and 1% β-mercaptoethanol were added to a vial and it was heated at 100 C for 5-10 min. To a well in the slab 15 µl of the standard solution were added.

Electrophoretic conditions

Electrophoresis was carried out using an LKB Model 2103 power supply. Electrophoresis was initially conducted at 25 mA until the samples entered the running gel. After

that a constant current of 45 mA was maintained.

After the electrophoresis run was completed the gel was removed from the electrophoretic chamber and clamped plates and it was soaked in the fixing solution for 90 min.

The slab gel was then stained for 90 min and destained for several hours or until clear. The gel was finally soaked in the 10% ethanol and 2% glycerol solution for 1 h.

After that it was dried on a Model SE-1150 Dual-temperature Slab Gel Dryer (Hoefer Scientific Instruments) for 1 h at 80 C.

A standard curve (Mw vs Rm) in semilogarithmic paper for the standard calibration protein mixture was prepared and it was used to determine the MW of the different bands.

Enzyme Study

Assay of Clotting Activity

The clotting activities of the various milk coagulants were determined at 30 C as described by Berridge (1945). The activity was determined by measuring the time required for 1 ml of the diluted enzyme to clot 10 ml of standard substrate at 30 C. The substrate was made by dissolving 12 g of pasteurized spray process Grade A low heat nonfat dry milk (Peake, Galloway West Co.) in 100 ml of 0.02 N $CaCl_2$.

The end point was observed by allowing a thin film of the milk to flow from a glass rod down the side of the 50 ml beaker containing the milk. When clotting occurred, the

almost invisible film broke into a number of white particles. The activity of the solution was then obtained from the formula:

$$\text{Rennin activity} = \frac{100\ d}{t}\ \text{Rennin units (R.U./ml)}$$

where d = dilution of the enzyme before adding to the milk and t = clotting time in sec.

Therefore a rennin unit was defined as the rennin activity which will clot 10 ml of the substrate in 100 seconds at 30 C.

After preparation of the substrate it was left for 1 h at room temperature before its use and it was used within 4 h after reconstitution.

Assay of Proteolytic Activity

The proteolytic activity of the coagulating enzymes was determined following a procedure described by Mickelsen and Fish (1970) with some modifications.

One milliliter of each of the enzyme solutions (five times more concentrated than used to get a clot in 5 min on Berridge's substrate) was added to 70 ml of Berridge substrate at 30 C. After 0, 5, 25 and 60 min intervals, 4 ml aliquots were removed from each solution and mixed with 4 ml of 24% trichloroacetic acid to precipitate the protein. The samples were filtered through Whatman No. 1 paper and the filtrate was collected for nonprotein nitrogen determination.

Digestion and distillation steps were conducted as for total nitrogen. Tests were done in duplicate.

Residual Enzyme Determination

A calcium caseinate-agar diffusion assay was used to measure the residual coagulant in curd after cooking, and in cheese during ripening. The method is based on one developed by Cheeseman (1963) and by Lawrence and Sanderson (1969) with some modifications. The materials were:

(1) Agar Noble (Difco Laboratories). Two grams were steamed with 100 ml deionized water. The liquid agar was then dispensed into test tubes in 12 ml aliquots and autoclaved 10 min.

(2) Bacto Isoelectric-Casein (Difco Laboratories). Two grams were dissolved in 100 ml 0.05 M Tris-maleate buffer pH 6.0 by heating to about 60 C.

(3) Calcium chloride, 0.01 M.

The procedure followed was:

(1) The 12 ml aliquots of agar were melted in the autoclave, cooled to 60 C, and kept at this temperature in a water bath.

(2) The casein solution was warmed to 60 C and 7 ml were added to each test tube containing the agar. One milliliter of 0.01 M calcium chloride was also added.

(3) After mixing thoroughly 2.5 ml aliquots were pipetted into 60x15 mm polystyrene disposable dishes to give a thickness of 1 mm.

(4) A well of 4 mm was drilled in the cooled agar with a No. 1 cork borer; the punched agar plugs were removed from the dishes by suction with a Pasteur pipette.

(5) The sample was added in the well using a Hamilton microsyringe. Three grams of cheese were mixed thoroughly with 15 ml distilled water. The pH of the cheese slurry was adjusted to 6.8 with 1.0 N NaOH and allowed to stand at room temperature (23 C) for 30 min. The slurry was then filtered through Whatman #2 filter paper and 5 µl of the filtrate were used for the enzyme analyses. In calculating the enzyme concentration in the cheese it was assumed that the enzyme was distributed uniformly in the aqueous phase of the curd.

(6) The plates were incubated at 37 C in moist atmosphere and removed after 20 h.

(7) The coagulant concentration was determined by measuring the diameter of the dense zone formed around the wells with a vernier caliper. The larger the zone, the greater the concentration of residual coagulant.

(8) Standard curves were prepared by plotting the logarithms of known concentrations of each enzyme against zone diameters.

Electrophoretic Pattern of Milk-coagulating Enzyme Preparations

For the electrophoretic run of the milk-coagulating enzyme preparations, the electrophoresis solutions were

prepared as described for cheese samples but in absence of urea. Samples of enzyme solutions in stacking gel buffer equivalent to approximately 7-10 μl of the commercial extract were used. A standard rennin from calf stomach (Sigma Chemical Co.) was also dissolved in stacking gel buffer and a sample with activity of 3 milk-clotting units was used (one unit coagulates 10 ml of milk per min at 30 C).

Electrophoresis was performed as for cheese samples with the following modifications:

a) A constant current of 3.00 mA per tube was applied.

b) Staining was done for 3 h.

Organoleptic Assessment

A panel of three experienced judges evaluated the cheeses for bitterness and cheese flavor. Samples were evaluated after 3, 6 and 9 months of ripening.

A piece of cheese of approximately 2 kg was presented to the judges for examination of the finish and general appearance. Three digit codes determined randomly were given to each cheese. A cheese trier was made available for removal of a plug of the cheese for testing.

The intensity of bitterness was scored in whole numbers on a scale of 1 to 6 according to Emmons *et al*. (1962):

1 = no detectable bitterness;

2 = barely detectable bitterness;

3 = distinct bitterness but at low level;

4 = moderate bitterness;

5 = very strong bitterness;

6 = unpalatable bitterness.

For the grading of cheese flavor a similar scale was used with the descriptions:

1 = no detectable cheese flavor;

2 = barely detectable cheese flavor;

3 = distinct cheese flavor, but at low level;

4 = substantial cheese flavor;

5 = strong cheese flavor;

6 = very strong cheese flavor.

A sample form is shown in Figure 2.

The same judges scored cheese flavor according to the ADSA procedure for Cheddar cheese. A flavor score of 10 required no criticism. The same score card was used to evaluate body and texture. A score of 5 required no criticism.

A sample form is shown in Figures 3 and 4.

Statistical Analysis

Data for composition of Cheddar cheese, composition of whey, and yield were analyzed by one way analysis of variance. If an F test proved significant, the Tukey multiple comparison was applied to determine significant differences among treatment means (Neter and Wasserman, 1974). Probability was 5%. A split plot design (Gill,

Name_____ CHEDDAR CHEESE EVALUATION Date_____

Please evaluate cheddar cheese samples ____, ____, ____, ____, ____, ____. Indicate your reaction to each characteristic by checking (✓) in the proper parenthesis.

Sample	OVERALL FLAVOR						Sample	BITTERNESS					
	—	—	—	—	—	—		—	—	—	—	—	—
No detectable cheese flavor	( )	( )	( )	( )	( )	( )	No detectable bitterness	( )	( )	( )	( )	( )	( )
Barely detectable cheese flavor	( )	( )	( )	( )	( )	( )	Barely detectable bitterness	( )	( )	( )	( )	( )	( )
Distinct cheese flavor but at low level	( )	( )	( )	( )	( )	( )	Distinct bitterness but at low level	( )	( )	( )	( )	( )	( )
Substantial cheese flavor	( )	( )	( )	( )	( )	( )	Moderate bitterness	( )	( )	( )	( )	( )	( )
Strong cheese flavor	( )	( )	( )	( )	( )	( )	Very strong bitterness	( )	( )	( )	( )	( )	( )
Very strong cheese flavor	( )	( )	( )	( )	( )	( )	Unpalatable bitterness	( )	( )	( )	( )	( )	( )

Comments about other characteristics of these samples?

Figure 2. Sample form of questionnaire presented to the experienced judges to evaluate bitterness and cheese flavor in experimental American Cheddar type cheese.

CONTEST

DATE _____ A.D.S.A. **CHEDDAR CHEESE SCORE CARD** D.I.S.A. _____ CONTESTANT NO. _____

PERFECT SCORE	CRITICISMS	SAMPLE NO. 1	2	3	4	5	6	7	8	9	10	TOTAL GRADE
FLAVOR	CONTESTANT SCORE ➧											
	GRADE SCORE											
	GRADE CRITICISM											
	ACID											
	BITTER											
NO CRITICISM 10	FEED											
	FERMENTED/FRUITY											
	FLAT/LACKS FLAVOR											
	GARLIC/ONION											
	HEATED											
	MOLDY											
NORMAL RANGE 1–10	RANCID											
	SULPHIDE											
	UNCLEAN											
	WHEY TAINT											
	YEASTY											
BODY AND TEXTURE	CONTESTANT SCORE ➧											
	GRADE SCORE											
	GRADE CRITICISM											
NO CRITICISM 5	CORKY											
	CRUMBLY											
	CURDY											
	GASSY											
NORMAL RANGE 1–5	MEALY											
	OPEN											
	PASTY											
	SHORT											
	WEAK											
COLOR	ALLOWED PERFECT IN CONTEST ➧	x	x	x	x	x	x	x	x	x	x	
FINISH	ALLOWED PERFECT IN CONTEST	x	x	x	x	x	x	x	x	x	x	
TOTAL	TOTAL SCORE OF EACH SAMPLE ➧											
	TOTAL GRADE PER SAMPLE											
											FINAL GRADE	
											RANK	

Figure 3. Sample form of questionnaire presented to experienced judges to evaluate flavor, body and texture of experimental American Cheddar type cheese.

Cheddar cheese

Flavor				Body and Texture			
Defect	S	D	P*		S	D	P
Acid	9	7	5	Corky	4	3	2
Bitter	9	7	4	Crumbly	4	3	2
Feed	9	8	6	Curdy	4	3	2
Fermented/fruity	8	6	5	Gassy	3	2	1
Flat/lacks flavor	9	8	7	Mealy	4	3	2
Garlic/onion	6	4	1	Open	4	3	2
Heated	9	8	7	Pasty	4	3	1
Moldy	7	5	3	Short	4	3	2
Rancid	6	4	1	Weak	4	3	2
Sulfide	9	7	4				
Unclean	8	6	5				
Whey taint	8	7	5				
Yeasty	6	4	1				

*S = denotes slight; D denotes definite; P denotes pronounced.

Figure 4. Suggested flavor, body and texture scores with designated intensities of flavor and body and texture defects for intercollegiate dairy products.

1978) was followed for moisture in time, pH in time, changes in pH 4.6-soluble nitrogen during ripening, and changes in 12% TCA-soluble nitrogen during ripening. The Tukey procedure was used to separate means at the 5% significance.

The split plot design is used when subjects are assigned randomly a treatment factor and then they are measured for trend at several sampling times. Therefore each observation is not measured on a different subject as in a completely randomized design.

A population model for the experiment is

$$Y_{ijk} = M + \alpha_i + D_{i(j)} + B_k + (\alpha B)_{ik} + (DB)_{(i)jk} + D_{(ijk)}$$

where $i = 1,...6$ $\quad j_{ijk}$ = observation

$j = 1,...4$

$k = 1,...5$

M is the overall mean.

The α_i are effects of the treatments (different coagulants) to which subjects (cheeses) are randomly assigned.

$D_{i(j)}$ represent random effects of subjects nested within treatments.

$B_{(k)}$ are the effects of time at the various sampling points (0, 1, 3, 6, and 9 months) in the process of repeated measurement of the subjects.

$(\alpha B)_{ik}$ is the interaction of treatment and time.

$(DB)_{(i)jk}$ is the interaction of subjects with time.

$E_{(ijk)}$ is the residual error within subjects.

The following Null hyoptheses were formulated for purpose of statistical analysis.

H_0^1: There are no significant differences between cheeses due to the different coagulants with regard to any of the dependent variables:

(a) moisture content

(b) pH

(c) pH 4.6-soluble nitrogen

(d) 12% TCA-soluble nitrogen

H_1^1: There are significant differences between cheeses due to the different coagulants with regard to the dependent variables previously mentioned.

H_0^2: There are no significant differences between cheeses due to time of ripening with regard to any of the dependent variables previously mentioned.

H_1^2: There are significant differences between cheeses due to time of ripening with regard to any of the dependent variables previously mentioned.

H_0^3: There are significant differences between cheeses due to interaction of treatments and time of ripening with regard to any of the dependent variables previously mentioned.

H_1^3: There are no significant differences between cheeses due to interaction of treatments and time of ripening with regard to any of the dependent

variables previously mentioned.

Data for sensory evaluation of cheese flavor and of bitterness were analyzed by a two factorial design where treatments (milk coagulants) and judges were the factors considered. If an F test proved significant, the Tukey multiple comparison was applied to determine significant differences among treatment means (Neter and Wasserman, 1974).

When variation in the experimental measurements of a variable was suspected to be caused by another variable a linear least squares regression analysis was performed.

RESULTS AND DISCUSSION

Composition of Experimental American Cheddar-Type Cheese

Typical composition of the experimental American Cheddar-type cheese made with the six coagulants is compared in Tables 2 and 3. Percent moisture, fat, total nitrogen, salt, ash content, fat in dry matter (FDM), moisture in the non-fatty substance (MNFS), and salt in moisture (S/M) are shown. The composition of all the cheese samples was similar. There was significant differences, however, between the ash content of the cheese made with Beemase (3.05%) and that of the cheese made with Marzyme II (3.55%). The moisture content ranged between 39.73% and 40.78%; the fat content between 30.20% and 31.75%; total nitrogen between 3.85% and 3.92%; salt content between 0.99% and 1.03%, and fat in dry matter (FDM) between 52.01% and 53.74%.

These results extend previous observations by Wong *et al*. (1977). They used calf rennet, rennet-pepsin blend, enzymes from *Actinomucor miehei* and *Actinomucor pusillus* var. *Lindt* to make Cheddar cheese and found no significant differences in the cheese composition due to the type of clotting enzyme.

Table 2. Percent composition of experimental American Cheddar-type cheese made with various coagulants.

Treatment	Moisture		Fat		Total Nitrogen		Salt		Ash	
	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE
					%					
Rennet	40.35	1.03[a]	31.37	1.42[a]	3.92	.11[a]	1.02	.06[a]	3.24	.15[a,b]
Emporase	40.78	.65[a]	31.73	1.23[a]	3.92	.02[a]	1.03	.05[a]	3.38	.10[a,b]
Beemase	39.73	.70[a]	32.30	1.36[a]	3.92	.07[a]	1.02	.02[a]	3.05	.04[a]
Econozyme	40.63	.17[a]	31.75	.86[a]	3.90	.04[a]	.99	.03[a]	3.40	.03[a,b]
Marzyme II	40.43	.66[a]	32.00	.48[a]	3.91	.02[a]	1.02	.05[a]	3.55	.07[b]
Bovin	40.62	.78[a]	30.20	.46[a]	3.85	.05[a]	1.01	.04[a]	3.45	.09[a,b]

[a,b]Means within a column not followed by a common letter differ ($p<.05$).

n=4

Table 3. Compositional factors of experimental American Cheddar-type cheese made with various coagulants.

Treatment	Fat in Dry Matter (FDM)		Moisture in the Non Fatty Substance (MNFS)		Salt in Moisture (S/M)	
	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE
			%			
Rennet	52.53	1.50[a]	58.79	.47[a]	2.55	.20[a]
Emporase	53.62	1.93[a]	59.77	1.06[a]	2.53	.13[a]
Beemase	53.53	1.67[a]	58.69	.35[a]	2.56	.09[a]
Econozyme	52.38	.61[a]	58.97	.28[a]	2.43	.06[a]
Marzyme II	53.74	1.00[a]	59.47	1.06[a]	2.53	.14[a]
Bovin	52.01	1.17[a]	58.74	.51[a]	2.49	.15[a]

[a]Means within a column not followed by a common letter differ ($P<.05$).

n=4

The Federal Standards of identity define Cheddar cheese as containing no more than 39.0% moisture, and not less than 50% of the fat as dry matter (FDM). Therefore the experimental cheese had appropriate FDM but slightly high moisture content. Many reasons can explain excess moisture in Cheddar cheese (Kosikowski, 1978): (1) the fat-to-casein levels are not in proper balance, (2) the curd is cut with wide wire knives, (3) the curds are not cooked sufficiently, (4) the curd blocks are not turned often enough during the Cheddaring step, (5) the blocks are piled double or triple instead of single, or (6) the salt content is low. In this study a combination of improper wire knives, fast acidity development that reduced the time of cooking and cheddaring, and low salt content may be the explanation for the slightly higher moisture content.

The speed of acidity development in the cheese vat affects the final cheese quality. A very rapid increase in lactic acid causes too much of the insoluble calcium phosphate to partition into the whey. This compound serves as an important buffer for maintaining satisfactory pH after salting; if it goes in the whey as soluble calcium lactate a low pH occurs at pressing (Wilster, 1974; Kosikowski, 1978). This may have occurred in the experimental cheese since there was a fast acidity development. This resulted, as will be shown further, in lower than normal pH in cheese throughout ripening. It may also have contributed

to the acid flavor reported in the sensory evaluation.

A normal practice in cheese manufacture is to add excess salt to cheese to compensate for the salt losses in whey in the vat and in the press. Since the quantity added to the cheese was exactly 1.5% the final concentration was around 1%, implying a loss of approximately 65% of the salt. According to Davies, Davis, Dearden, and Mattick (1937) salt in cheese may influence the following: 1) the inhibition of certain types of microorganisms, 2) the activation of proteolytic enzymes of rennet, 3) the solubilization of certain proteins or protein degradation compounds, 4) the acid-base equilibria in the cheese, 5) the rate of loss of whey (moisture and soluble constituents), and 6) the proportion of bound and free water. They found that the omission of the salt resulted in 50% increase in ripening rate as measured by protein degradation. Apparently, the low salt content slightly increased moisture, and low pH throughout ripening may have had some adverse influence in the flavor and texture of the cheese.

Moisture in the non-fatty substance (MNFS) represents the ratio of water to protein in cheese. Salt in moisture (S/M) represents the ratio of salt to moisture. Lawrence and Gilles (1980, 1982) in New Zealand have identified these factors together with fat in dry matter (FDM) as being critical in determining the quality of Cheddar cheese. Accordingly, corresponding values for MNFS, S/M and FDM

were calculated.

Cheese Yield

The recovery of milk solids (yield) is affected not only by the parameters of cheese manufacture but also by the milk coagulant utilized. Christensen (1979) indicated that one of the factors affecting yield was the firmness of the curd at the time of cutting, which varies for different coagulants. Foe example, a slow or weak set may cause a high percent of whey fat and consequently a low yield. In addition, protein losses in whey are positively correlated to a higher proteolytic activity or to less specificity of the enzymes used as coagulants (Krishna-Rao et al., 1979). In contrast, it has also been reported that cheese producers have experienced little difficulty in achieving comparable cheese yields with various commercial milk clotting enzymes (Nelson, 1975).

The yield and average amounts of fat and protein recovered in the experimental cheese are shown in Table 4. Cheese yields were higher for rennet, followed by Emporase, Bovin, Econozyme, Marzyme II, and Beemase. These differences were not, however, statistically significant. The values ranged from 9.62% for rennet to 9.35% for Beemase and are in agreement with the normal yields reported in the literature.

Table 4. Yield and average amounts of fat and protein recovered from experimental American Cheddar-type cheese made from 100 kg of milk.

Treatment	Yield		Recovery of Fat		Recovery of Protein[1]	
	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE
			%			
Rennet	9.62	.14[a]	92.01	2.33[a]	69.45	1.11[a,b]
Emporase	9.46	.16[a]	91.61	2.17[a]	69.44	.77[a,b]
Beemase	9.35	.13[a]	92.16	2.77[a]	70.38	.27[a]
Econozyme	9.40	.12[a]	91.23	2.80[a]	68.64	.59[a,b]
Marzyme II	9.39	.11[a]	91.91	2.09[a]	67.71	.51[a,b]
Bovin	9.44	.12[a]	87.20	1.65[a]	67.02	.48[b]

[1]Total protein = total nitrogen x 6.38

[a,b]Means within a column not followed by a common letter differ ($P<.05$).

n=4

Yield studies utilizing small vats are generally not considered accurate for drawing conclusions for commercial practices (Green et al., 1975). The small losses of curds in small batches can be significant when transferred to a large operation. Only sufficient volumes of milk will allow accurate determination of the comparative effects of a specific ingredient such as the clotting enzyme (Sellars, 1982). Possibly the use of small vats in this study may not discern yield differences. Although the differences were not statistically significant they could be economically and commercially significant. Also, similar yields obtained with each treatment agree with the results reported by Nelson (1975).

A fraction of milk fat and protein are always lost in the whey during cheesemaking. Thus, whey fat and protein content can be indicators of cheese yield. Table 5 presents percentage solids, fat, and nitrogen of whey obtained during manufacture of the experimental cheese. Again, there was no significant difference in solids, fat or nitrogen content. Solids fluctuated from 6.27% to 6.74%; fat from 0.30% to 0.35%; nitrogen from 0.12% to 0.14%. Van Slyke (1979) reported an average of 6.96% milk solids, 0.36% fat, and 0.13% nitrogen in whey at Cheddar cheese factories in New York.

Table 5. Percent composition of whey obtained during manufacture of experimental American Cheddar-type cheese made with various coagulants.

Treatment	Solids		Fat		Nitrogen	
	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE
			%			
Rennet	6.74	.19[a]	.30	.03[a]	.13	.00[a]
Emporase	6.72	.15[a]	.31	.04[a]	.13	.00[a]
Beemase	6.55	.26[a]	.32	.04[a]	.14	.00[a]
Econozyme	6.66	.18[a]	.34	.03[a]	.13	.00[a]
Marzyme II	6.43	.24[a]	.35	.02[a]	.12	.00[a]
Bovin	6.27	.29[a]	.35	.02[a]	.13	.00[a]

[a]Means within a column not followed by a common letter differ ($P<.05$).

n=4

Changes in Moisture Content as a Function of Time

Cheese is waxed or film wrapped to protect it from microbial contamination and oiling-off, and to reduce the rate of moisture loss. Control of moisture is an important factor in quality control because it affects ripening rate, pH, flavor and texture of cheese. In this study, cheese was vacuum-packaged in Cryovac bags. Moisture content was determined after 0, 1, 3, 6, and 9 months of ripening. The results are shown in Table 6 and illustrated in Figure 5.

In all cases there was no statistically significant change in moisture as a function of time of ripening, as a function of treatment, i.e., the milk coagulant used, or because of time treatment interaction. The relatively small changes in moisture content observed may be due to the opening of the cheese packages for sample collection at the different time points, rather than because of failure of the Cryovac bag to work as an effective barrier. Individual packages for each time point would have been ideal.

Changes in pH During Ripening

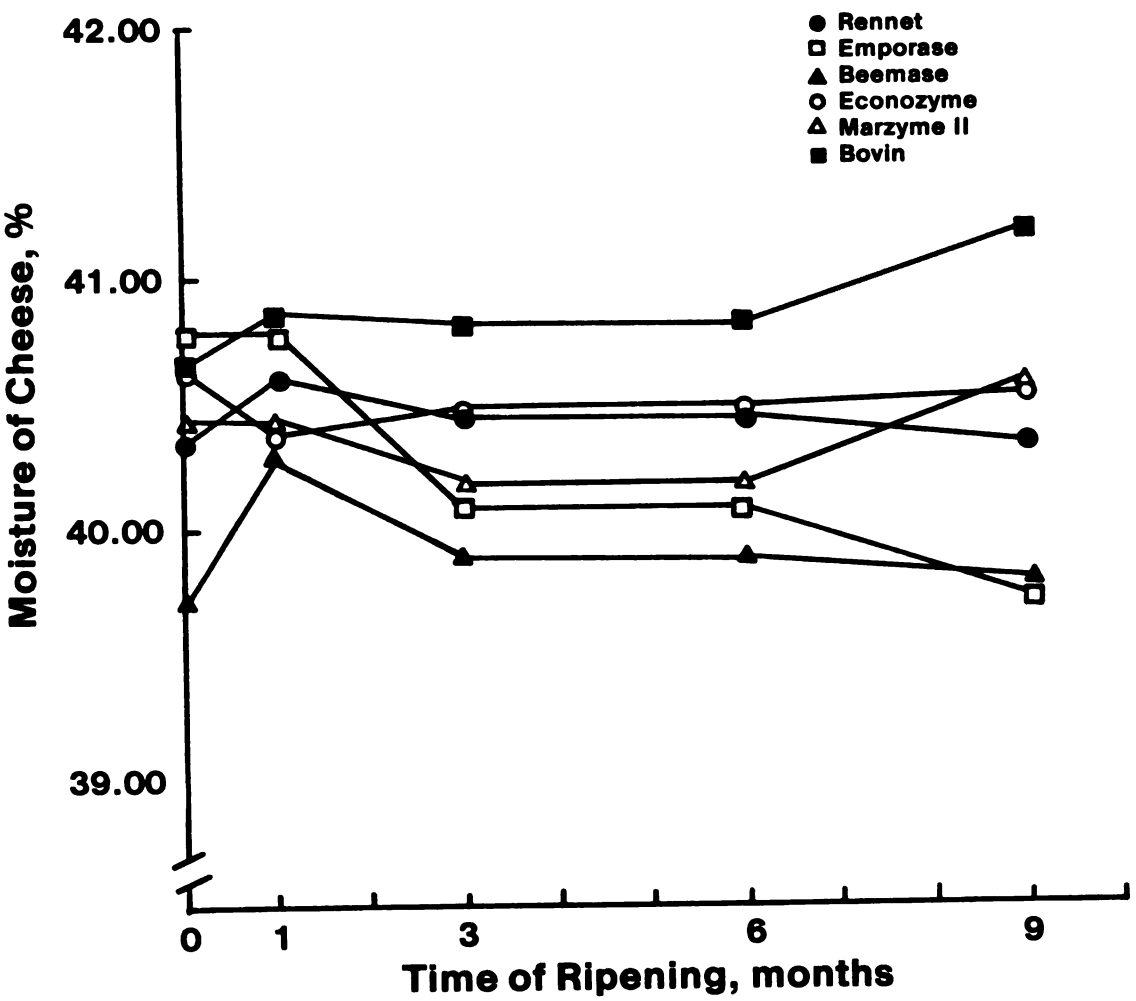
Cheese pH measurements are important because pH influences the activity of the enzyme systems involved in ripening. Normally there is a gradual increase in pH of

Table 6. Average changes in moisture content during ripening of experimental American Cheddar-type cheese made with various coagulants[1].

Treatment	Time of Ripening (months)										Overall Mean
	0		1		3		6		9		
	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE	
	%										
Rennet	40.35	1.03	40.60	.78	40.44	.80	40.44	.80	40.33	.80	40.43
Emporase	40.78	.65	40.77	.32	40.06	.63	40.06	.63	39.71	.51	40.28
Beemase	39.73	.70	40.29	.35	39.88	.45	39.88	.45	39.79	.52	39.91
Econozyme	40.63	.17	40.35	.31	40.46	.28	40.46	.28	40.51	.41	40.48
Marzyme II	40.43	.66	40.43	.79	40.18	.64	40.18	.64	40.53	.61	40.35
Bovin	40.62	.78	40.86	1.01	40.81	.91	40.81	.91	41.19	1.05	40.86

[1]Treatment, time, and interaction are not significant ($P<.05$), n=4.

Figure 5. Average changes in moisture content during ripening of experimental American Cheddar-type cheese made with various coagulants.


Rennet
Emporase
Beemase
Econozyme
Marzyme II
Bovin
42.00
41.00
40.00
39.00
Moisture of Cheese, %
0
1
3
6
9
Time of Ripening, months

most cheeses during ripening. This is caused by destruction of the lactic acid, formation of non-acidic transformation products, and appearance of weaker or less dissociated acids, such as acetic and carbonic. Liberation of alkaline products of protein decomposition contribute further to pH rise (Ernstrom and Wong, 1974). Cheddar cheese pH decreases to between 4.95 and 5.0 during the first few days; it increases only slightly for a few months thereafter, finally increasing more rapidly to approximately 5.3 in one year.

pH data for experimental cheese (Table 7, Figure 6) indicate that trends were similar to a normal cheese but the values were lower. Rapid acid development during cheese manufacture may have caused lower pH values.

Starter used in the manufacture of the experimental cheese was DVS Redi-Set frozen lactic culture. This culture comes in 360 ml cans designed for 2265 kg of milk. Although the proper calculations were made to adjust to small vats, it is possible that too much culture was used.

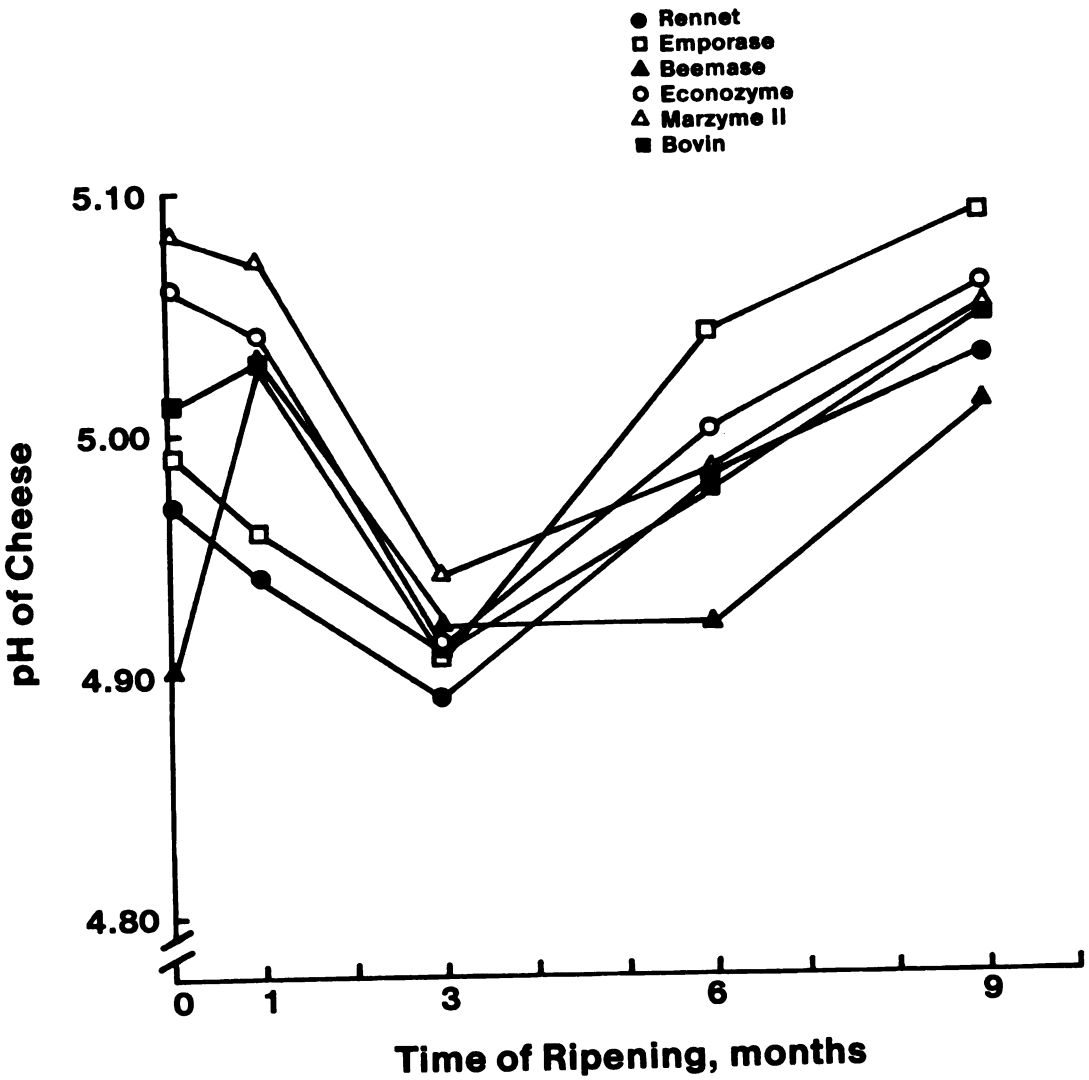
In general, pH decreased after 3 months, followed by a steady increase up to 9 months of ripening. No changes in pH due to coagulant used or to time-coagulant interaction were significant. However, time produced significant changes in pH value.

Table 7. Average changes in pH during ripening of experimental American Cheddar-type cheese made with various coagulants.

Treatment	Time of ripening (months)										Overall Mean
	0		1		3		6		9		
	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE	
Rennet	4.97	.05	4.94	.03	4.89	.02	4.98	.06	5.03	.05	4.96
Emporase	4.99	.05	4.96	.04	4.91	.02	5.04	.02	5.09	.03	5.00
Beemase	4.90	.01	5.03	.05	4.92	.03	4.92	.03	5.01	.02	4.96
Econozyme	5.06	.04	5.04	.05	4.91	.02	5.00	.02	5.06	.03	5.01
Marzyme II	5.08	.05	5.07	.09	4.94	.08	4.98	.06	5.05	.04	5.02
Bovin	5.01	.05	5.03	.07	4.91	.05	4.98	.03	5.05	.04	5.00

[1]Time is significant ($P<.05$). Treatment and interaction are not significant ($P<.05$), n=4.

Figure 6. Average changes in pH during ripening of experimental American Cheddar type cheese made with various coagulants.


Rennet
Emporase
Beemase
Econozyme
Marzyme II
Bovin
5.10
5.00
4.90
4.80
pH of Cheese
0
1
3
6
9
Time of Ripening, months

Changes in Protein During Ripening

Proteolysis in cheese during ripening is governed by four main proteolytic agents; milk coagulant, starter bacteria and their enzyme, non-starter bacteria and their enzymes, and endogeneous milk proteases. In this work, comparative action of various coagulants was investigated by fixing other variables involved in proteolysis. Accordingly, starter culture, source of milk, manufacturing schedule and technique were kept constant in all trials.

Formation of pH 4.6-Soluble Nitrogen and 12% TCA-Soluble Nitrogen

Practically all of the nitrogenous constituents of young cheese exist as water insoluble protein. As ripening progresses part or all of the protein is hydrolyzed enzymatically to simpler, water-soluble compounds (Foster, Nelson, Speck, Doetsch, and Olson, 1957). As an index of cheese ripening distribution of nitrogen among a number of fractions may be characterized by such properties as solubility in 12% TCA, solubility at pH 4.6, solubility in 70% ethanol, precipitability by calcium ions. These changes in nitrogen distribution determine rate of proteolysis of casein (Green, 1977).

According to O'Keeffe *et al*. (1978) Cheddar cheese protein undergoes extensive degradation during ripening.

Approximately 30% and 5% of the total protein in mature cheese is soluble at pH 4.6 and 12% TCA, respectively. Reville et al. (1978) report values of 26% and 19% for pH 4.6 and 12% TCA-soluble nitrogen of 12-month old Cheddar cheese. The nitrogen soluble in pH 4.6 buffers consists predominantly of large, medium and small peptides. The 12%-TCA soluble nitrogen represents mostly the non-protein nitrogen (NPN).

Rates of formation of pH 4.6-soluble nitrogen in experimental cheese made with the six different coagulants as a function of time are shown in Table 8 and Figure 7.

At 0 time level of pH 4.6-soluble nitrogen expressed as a percentage of the total nitrogen was approximately 7 to 7.5% for cheese made with Emporase, Beemase, Rennet and Bovin. Cheese made with Marzyme II and Econozyme showed values of about 5%. After 1 month of ripening the levels rose to about 16% for cheese made with Emporase and with Beemase; to about 12.5% for cheese made with Rennet and with Bovin, and to about 10% for cheese made with Marzyme II and with Econozyme. Statistically, the increases from 0 to 1 month of ripening were significantly different due to time of ripening but not due to the different coagulants used.

After 3 months increases in pH 4.6-soluble nitrogen became significant due to the coagulant used, time of ripening and coagulant-time interaction. Cheese made with

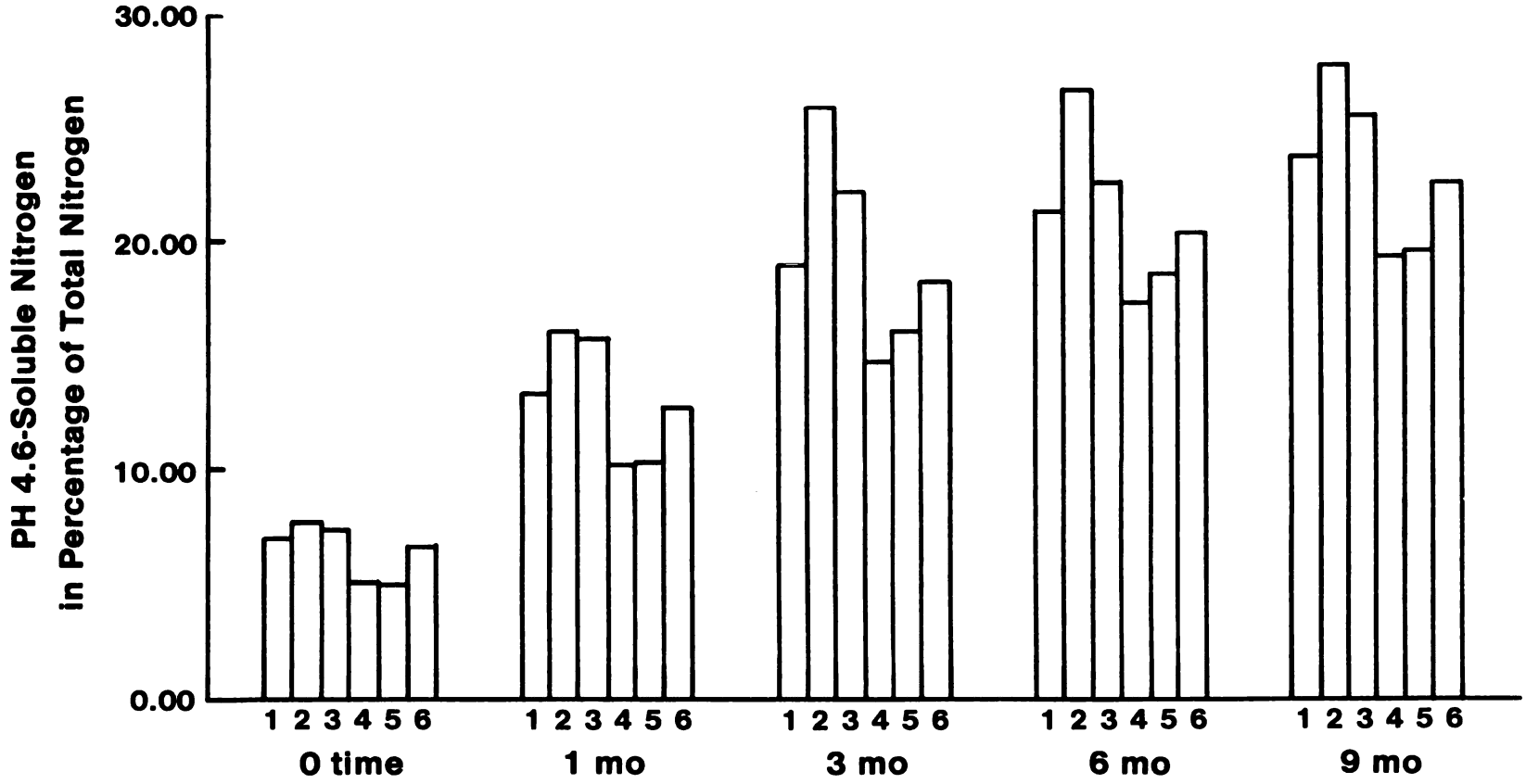
Table 8. Average changes in pH 4.6-soluble nitrogen during ripening of experimental American Cheddar type cheese made with various coagulants.

Treatment	Time of Ripening (months)										Overall Mean
	0		1		3		6		9		
	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE	
	pH 4.6 soluble nitrogen, g per 100 g cheese[1]										
Rennet	.27	.01[a]	.52	.01[a]	.74	.02[a,c]	.84	.03[a,b]	.93	.04[a,b,c]	.66
Emporase	.30	.04[a]	.64	.06[a]	1.03	.04[b]	1.06	.05[b]	1.11	.03[b]	.83
Beemase	.30	.02[a]	.63	.01[a]	.89	.02[b,c]	.90	.03[a,b]	1.02	.05[a,b]	.75
Econozyme	.20	.03[a]	.41	.02[a]	.59	.01[a]	.69	.04[a]	.77	.05[a,c]	.53
Marzyme II	.20	.00[a]	.40	.04[a]	.63	.05[a]	.73	.06[a]	.77	.07[c]	.55
Bovin	.25	.02[a]	.49	.02[a]	.70	.02[a,c]	.79	.02[a]	.87	.03[a,b,c]	.62

[a,b,c]Means within a column not followed by a common letter differ ($P<.05$).

[1]Treatment, time and interaction were significant ($P<.05$), n=4.

Figure 7. Development of pH 4.6-soluble nitrogen during ripening of experimental American Cheddar-type cheese made with various coagulants: 1=rennet; 2=Emporase; 3=Beemase; 4=Econozyme; 5=Marzyme II; 6=Bovin.


PH 4.6-Soluble Nitrogen
in Percentage of Total Nitrogen
30.00
20.00
10.00
0.00
1 2 3 4 5 6
0 time
1 2 3 4 5 6
1 mo
1 2 3 4 5 6
3 mo
1 2 3 4 5 6
6 mo
1 2 3 4 5 6
9 mo

Emporase showed about 1.03% soluble nitrogen or 26% of the total nitrogen. This level was higher and significantly different than the level observed in cheese made with Marzyme II, Bovin, and Econozyme. It was not different from that of cheese made with Beemase and rennet. Cheese made with Beemase had 0.89% soluble nitrogen or about 23% of the total nitrogen soluble at pH 4.6. Cheese made with rennet had 0.74% soluble nitrogen or about 19% of the total nitrogen soluble at pH 4.6. Cheese made with Bovin had 0.70% soluble nitrogen or about 18% of the total nitrogen soluble at pH 4.6. Lowest levels were observed for cheese made with Marzyme II (0.63% or 16% of the total nitrogen) and with Econozyme (0.59% or 15% of the total nitrogen).

After 6 and 9 months of ripening the levels continued to increase. The cheese made with Emporase continued to have the highest level of pH 4.6-soluble nitrogen (28% of the total nitrogen). Cheeses made with Beemase, rennet, and Bovin had 26%, 23.7% and 22.5%, respectively. Lowest levels (approximately 19.7%) were those of cheese made with Econozyme and Marzyme II.

Development of 12% TCA-soluble nitrogen is shown in Table 9 and Figure 8. After 0, 1, 3, and 6 months of ripening there was no significant difference in the levels of 12% TCA-soluble nitrogen due to treatment, i.e., due to the coagulant used. After 9 months, however, the coagulant used had a significant effect on the increase of 12%

Table 9. Average changes in 12% TCA-soluble nitrogen during ripening of experimental Cheddar-type cheese made with various coagulants.

Treatment	(Time of Ripening (months)										Overall Mean
	0		1		3		6		9		
	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE	
	12% TCA soluble nitrogen, g per 100 g cheese[1]										
Rennet	.14	.00[a]	.31	.01[a]	.43	.01[a]	.53	.03[a]	.53	.03[a,b,c]	.41
Emporase	.14	.01[a]	.30	.01[a]	.49	.04[a]	.58	.04[a]	.71	.08[a,c]	.44
Beemase	.16	.01[a]	.34	.02[a]	.53	.04[a]	.59	.03[a]	.72	.05[c]	.47
Econozyme	.08	.01[a]	.20	.02[a]	.35	.02[a]	.40	.03[a]	.51	.03[a,b]	.31
Marzyme II	.08	.01[a]	.20	.02[a]	.38	.02[a]	.39	.05[a]	.49	.08[b]	.31
Bovin	.10	.01[a]	.21	.03[a]	.42	.05[a]	.43	.03[a]	.54	.04[a,b,c]	.34

[a,b,c]Means within a column not followed by a common letter differ ($P<.05$).

[1]Treatment and time were significant ($P<.05$). Interaction was not significant. n=4.

Figure 8. Development of 12% TCA-soluble nitrogen during ripening of experimental American Cheddar-type cheese made with various coagulants; 1=rennet; 2=Emporase; 3=Beemase; 4=Econozyme; 5=Marzyme II; 6=Bovin.

12% TCA-Soluble Nitrogen
in Percentage of Total Nitrogen
20.00
10.00
0.00
1 2 3 4 5 6
0 time
1 2 3 4 5 6
1 mo
1 2 3 4 5 6
3 mo
1 2 3 4 5 6
6mo
1 2 3 4 5 6
9 mo

TCA-soluble nitrogen. At this time cheese made with Beemase showed the highest level (18% of the total nitrogen) followed by Emporase, rennet, and Bovin which had 18%, 16.25%, and 14%, respectively. Lowest levels were shown by cheese made with Econozyme (13%) and Marzyme II (12.5%). Beemase was significantly different from Marzyme II and Econozyme; Emporase was different only from Marzyme II.

O'Keeffe et al. (1976, 1978) proposed that proteolysis as measured by changes in electrophoretic patterns and formation of pH 4.6-soluble nitrogen compounds is due mainly to the coagulant. On the other hand, the formation of small peptides and amino acids is principally due to the activity of starter peptidases. Furthermore Petterson and Sjostrom (1975) showed a direct relationship between starter cell numbers and levels of 12% TCA-soluble nitrogen in Swedish semihard cheese. Visser (1977b) studied protein degradation in aseptically made Gouda cheese and concluded that rennet was the dominant agent for the production of peptides of high, medium and low molecular weight during ripening of cheese. Conversely, starter bacteria were responsible for amino acid production. Emmons et al. (1962) prepared Cheddar cheese using different strains of starter culture. Different strains gave no significant differences in acid-soluble nitrogen. But significant differences in TCA-soluble nitrogen, and highly significant differences in amino nitrogen were related to strain differences.

In this work, the coagulant significantly affected level of pH 4.6-soluble nitrogen after 3, 6, and 9 months of ripening. Levels of 12% TCA-soluble nitrogen were significantly affected by the type of coagulant used, but only after 9 months of ripening. These results suggest that the coagulant is largely responsible for proteolysis of the caseins to large and medium size peptides, while starter is mostly responsible for the NPN development.

In closing, using pH 4.6- and 12% TCA-soluble nitrogen as criteria for proteolysis, Emporase and Beemase showed the highest proteolytic activity. Emporase is a preparation from Actinomucor pusillus and Beemase is a mixture of Actinomucor pusillus protease and bovine pepsin. An intermediate proteolytic activity was shown by rennet and Bovin. Marzyme II (Actinomucor miehei preparation) and Econozyme (mixture of rennet and porcine pepsin) were least proteolytic. The fact that Econozyme had lower proteolytic activity than rennet may be due to relative thermolability of pepsin (Green, 1972).

Table 10 shows percentage of 12% TCA-soluble nitrogen expressed as a percentage of the pH 4.6-soluble nitrogen. As time increased, the percentage increased indicating further degradation of large and medium peptides to smaller peptides and amino acids which are soluble in 12% TCA.

It is interesting to note that cheese showing highest levels of soluble nitrogen fractions had lowest overall

Table 10. Percentage 12% TCA-soluble nitrogen expressed as a percentage of the pH 4.6 soluble nitrogen as a function of time.

Treatment	Time of Ripening, months				
	0	1	3	6	9
			%		
Rennet	51.85	59.62	58.11	63.1	67.74
Emporase	46.66	46.87	47.57	54.72	63.96
Beemase	53.33	53.97	59.55	65.55	70.59
Econozyme	40.00	48.78	59.32	57.97	66.23
Marzyme II	40.00	50.00	60.32	53.42	63.36
Bovin	40.00	42.86	60.00	54.43	62.06

pH. It is therefore possible that low pH may have accelerated proteolysis.

Discontinuous Gel Electrophoresis

Gel electrophoretic methods are increasingly used to study the nature and extent of casein degradation in cheese.

In this study DISC-PAGE was used to compare the proteolytic action of different coagulants in Cheddar cheese. Changes in electrophoretic characteristics in specific casein components during ripening were investigated. Quantitation was attempted by densitometric scanning of the gels. From the densitometric patterns the proportions of α_{S1}- and β-casein were calculated in comparison with the proportions present in samples of freshly made cheese. Because of the large number of resolved zones, especially as the time of ripening increased, specific identification of any zone was difficult to establish. However, by the shape of the densitogram and relative mobility, certain zones were followed during the course of ripening. Only α_{S1}- and β-casein could be almost certainly identified by comparison with the electrophoretic pattern and densitogram of whole casein.

Visser et al. (1977) from electrophoretic studies on aseptically made Gouda cheese concluded that rennet was completely responsible for the observed degradation of

α_{S1}-casein and for the decomposition of β-casein during the first months of ripening. In the longer term, starter bacteria also appeared to contribute especially in further degradation of β-casein. In agreement, O'Keeffe et al. (1976, 1978) stated that rennet was the main agent responsible for the level of proteolysis detected by gel electrophoresis.

The densitometric pattern of an electrophoretic gel of whole casein is shown in Figure 9.

In Figure 10 electrophoretic patterns and facsimilies of experimental cheese made with calf rennet at different times of ripening are presented. It appears that α_{S1}-casein (identified with the number 7) was degraded extensively early in the ripening process. The densitometric readings (Figure 11, Table 11) show that after 1 month of ripening the α_{S1}-casein is about 35.4% of the original quantity; after 9 months only about 14.5% remains. The α_{S1}-casein was degraded primarily into a produce with slightly higher mobility; most probably α_{S1}-I (Visser et al., 1977). This product increased from 0 to 1 month, subsequently hydrolyzing to smaller, faster compounds, possibly similar to those described by Mulvihill and Fox (1977). β-Casein degradation was slower; after 1 month 94.4% was intact; after 9 months about 70% of the original quantity remained. Zones between α_{S1}- and β-casein were observed at all times, some increasing and others decreasing (identified with

Figure 9. DISC-PAGE pattern of whole casein and its densitogram.

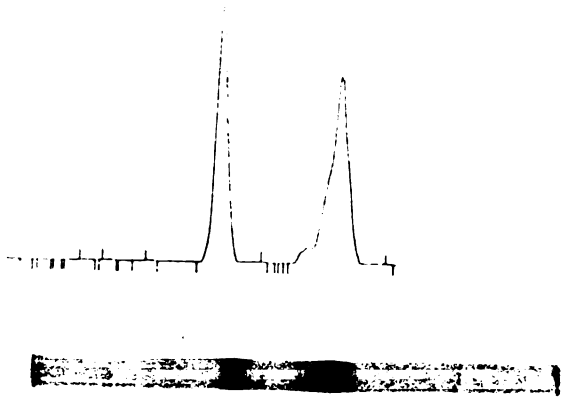
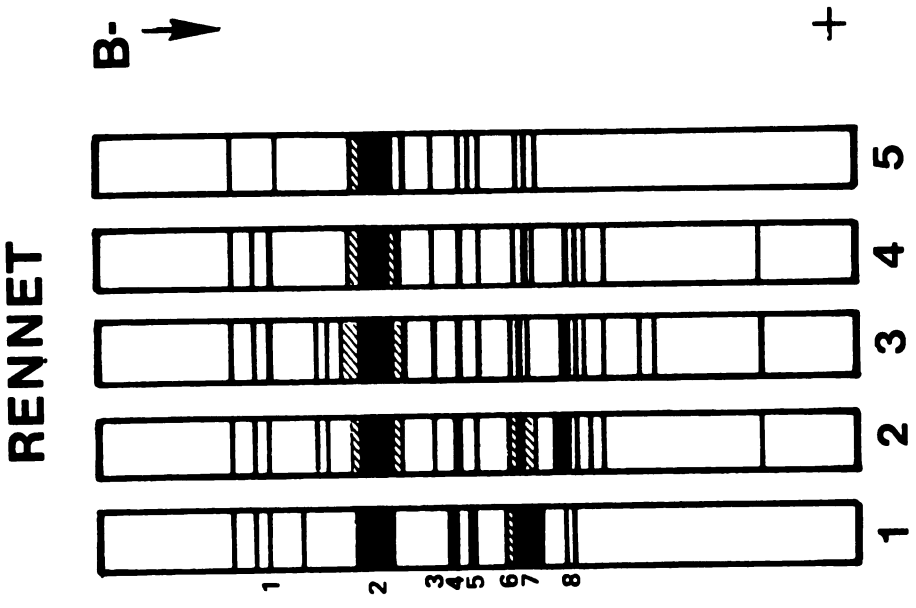


Figure 10. DISC-PAGE (9% T) patterns and facsimilies of experimental American Cheddar type-cheese made with rennet, after 0 time of ripening (1-A, 1-B), 1 month (2-A, 2-B), 3 months (3-A, 3-B), 6 months (4-A, 4-B), and 9 months (5-A, 5-B).


B-
+
RENNET
5
4
3
2
1
1
2
3
4
5
6
7
8

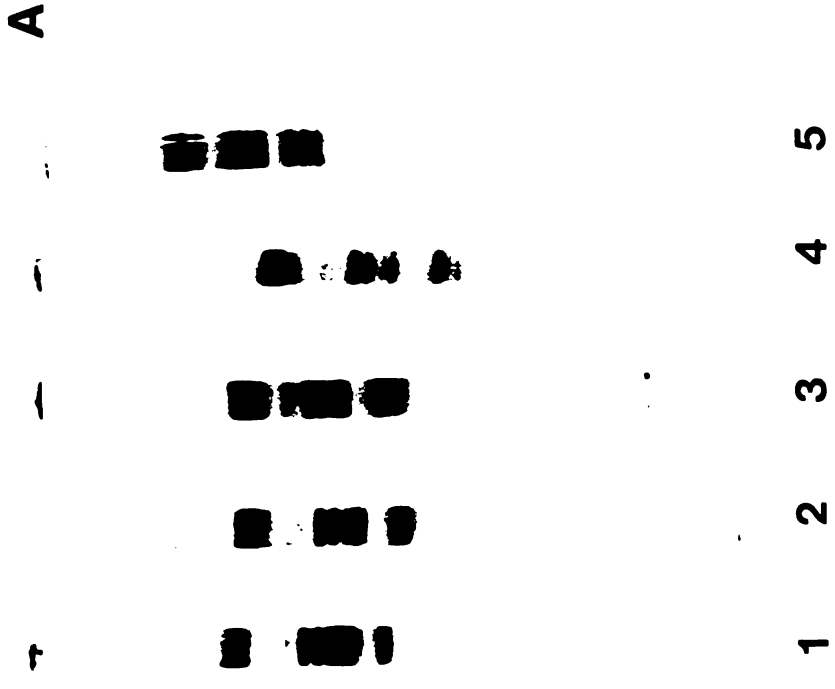

A
5
4
3
2
1

Figure 11. Densitometric scanning patterns of electrophoresis gels of experimental American Cheddar type cheese made with calf rennet, after 0, 1, 3, 6, and 9 months of ripening.

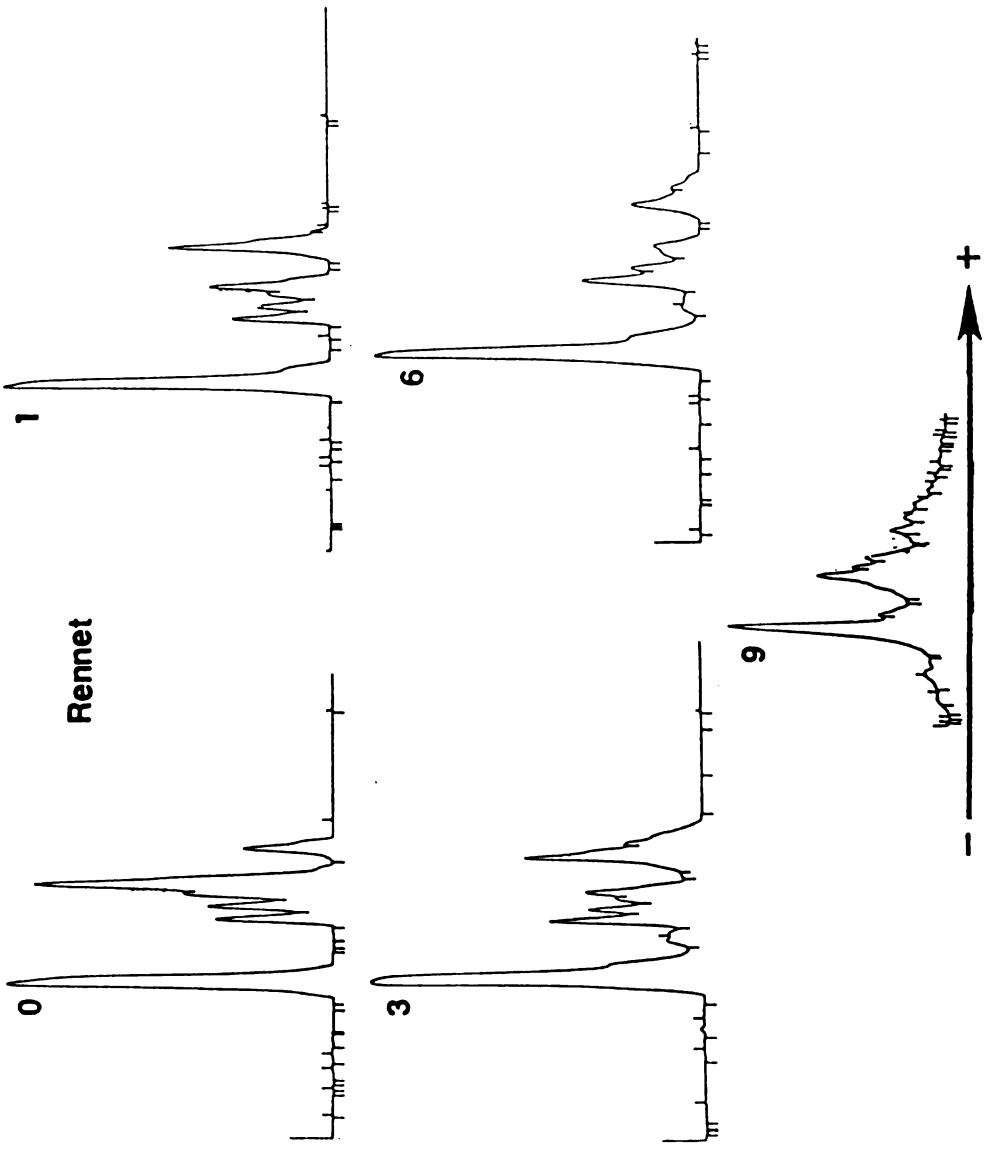

Rennet
0
1
3
6
9
–
+

Table 11. Relative percentage of original α_{S1}- and β-casein fractions at different times of ripening.

Treatment		Time of ripening, months							
		1		3		6		9	
		$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE
		Relative percentage of original casein fractions[1]							
Rennet	α_{S1}	35.4	7.2	24.2	3.9	18.4	2.7	14.5	2.1
	β	94.4	3.3	103.2	6.8	94.0	13.8	70.0	10.8
Emporase	α_{S1}	91.4	1.4	76.5	11.4	53.9	10.2	MP*	MP
	β	71.5	10.7	68.2	9.7	62.3	7.1	MP	MP
Beemase	α_{S1}	43.6	10.4	33.7	3.7	23.4	2.6	16.5	0.0
	β	82.2	6.2	76.6	4.2	58.5	4.8	43.8	0.0
Econozyme	α_{S1}	44.4	22.6	20.0	10.3	22.0	1.8	17.1	1.4
	β	109.5	9.5	93.0	13.2	86.2	10.3	71.6	8.3
Marzyme II	α_{S1}	42.3	12.6	51.5	8.0	41.3	6.5	11.6	1.2
	β	81.8	.4	76.9	0.5	83.9	1.9	50.0	0.0
Bovin	α_{S1}	26.6	7.7	17.0	5.0	13.7	1.8	12.5	4.1
	β	83.9	21.	72.0	14.5	73.8	1.8	60.2	2.4

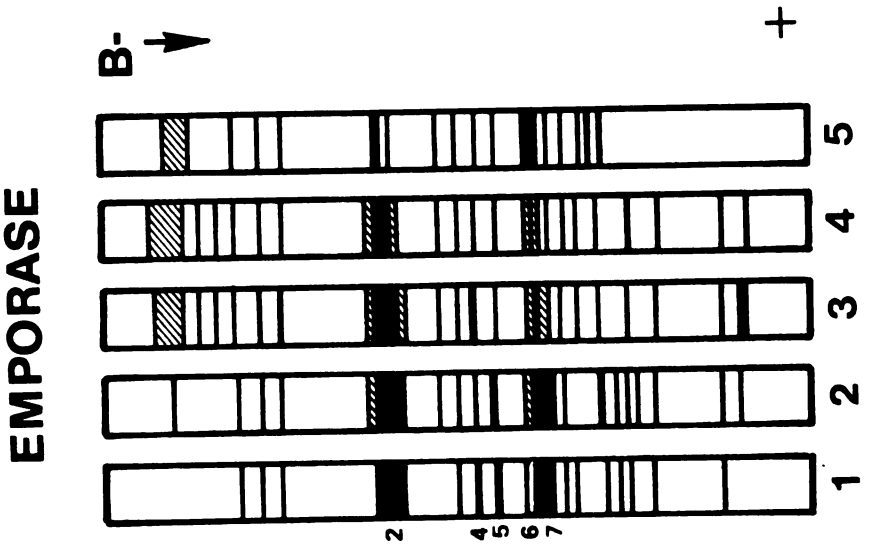
[1]Numbers were obtained by dividing the area of each peak by its area at 0 time of ripening. n=3. *MP = Merged peak, impossible to identify.

numbers 4 and 5); they could be products of degradation of β-casein (B-I, B-II, B-III) previously described by Creamer et al. (1971). The higher degradation of α_{S1}-casein compared to β-casein is in agreement with the work of Ledford et al. (1966). These workers used polyacrylamide gel electrophoresis to show that in Cheddar cheese made with rennet, α_{S1}-casein was extensively degraded while β-casein underwent only slight proteolysis.

Figure 12 represents patterns and facsimilies of experimental cheese made with Emporase. α_{S1}-Casein was also degraded but this time only after 3 months of ripening. Instead of just one, a number of products of higher electrophoretic mobility resulted. β-Casein was degraded to a greater degree by the Actinomucor pusillus preparation than by rennet. The densitometric readings (Figure 13, Table 11) showed that after one month 71.5% was left; after 6 months about 62% of the β-casein was intact, and after 9 months of ripening the densitometric pattern was so degraded that it was impossible to identify α_{S1}- or β-casein with certainty. In general, Emporase seemed to degrade α_{S1}-casein slower than rennet, and β-casein at comparable or slightly higher rate. These results are comparable to the works of Edwards and Kosikowski (1969) and Vanderpoorten and Weckx (1972).

Figure 14 represents patterns and facsimilies of experimental cheese made with Beemase. Again, α_{S1}-casein

Figure 12. DISC-PAGE (9% T) patterns and facsimilies of experimental American Cheddar-type cheese made with Emporase, after 0 time of ripening (1-A, 1-B), 1 month (2-A, 2-B), 3 months (3-A, 3-B), 6 months (4-A, 4-B), and 9 months (5-A, 5-B).


EMPORASE
B-
+
5
4
3
2
1
2
4
5
6
7

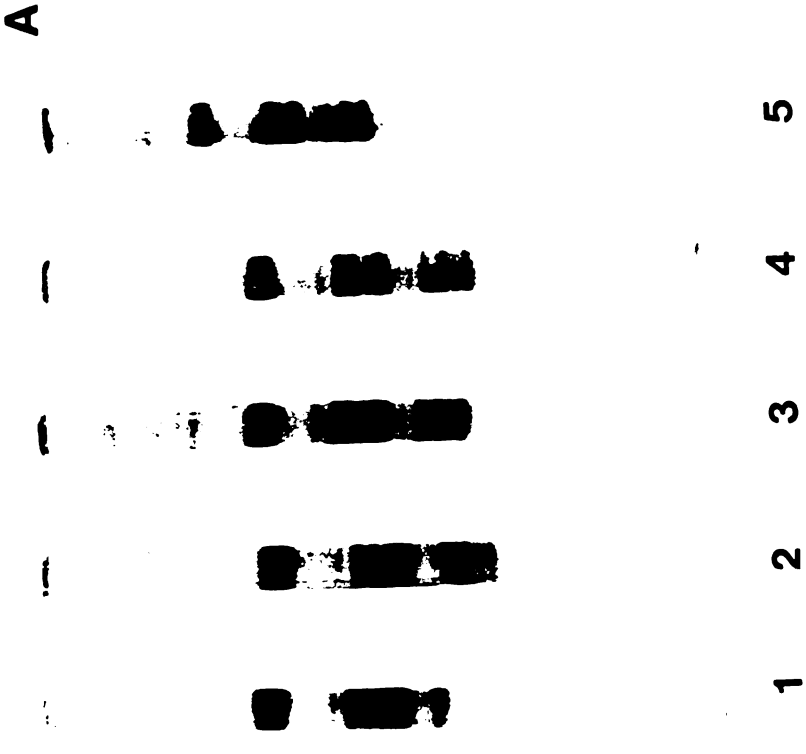
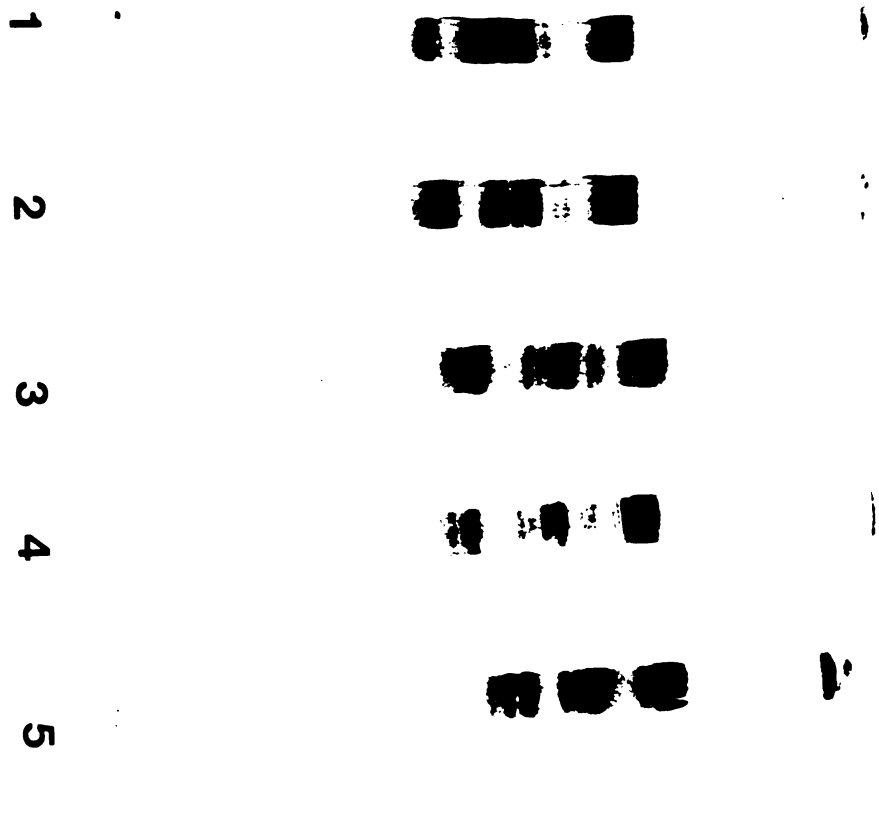
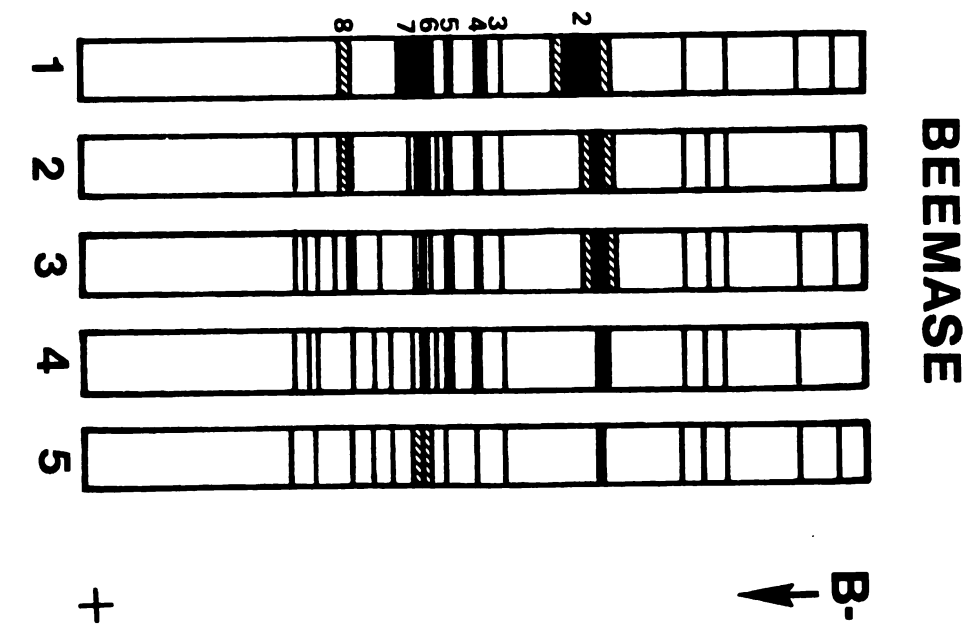

A
5
4
3
2
1

Figure 13. Densitometric scanning patterns of electrophoresis gel of experimental American Cheddar type cheese made with Emporase, after 0, 1, 3, 6 and 9 months of ripening.

Figure 14. DISC-PAGE (9% T) patterns and facsimilies of experimental American Cheddar-type cheese made with Beemase, after 0 time of ripening (1A, 1B), 1 months (2-A, 2-B), 3 months (3-A, 3-B), 6 months (4-A, 4-B) and 9 months (5-A, 5-B).


1
2
3
4
5
A

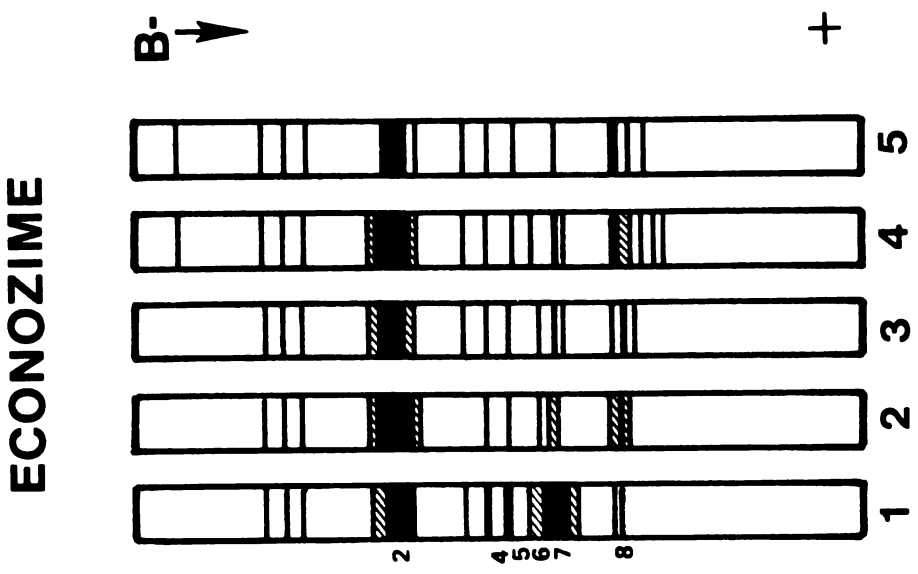

8
7
6
5
4
3
2
1
2
3
4
5
BEEMASE
+
B

was degraded to a product of higher mobility (presumably α_{S1}-I) which in time was degraded to two smaller products. As in the case of cheese made with Emporase β-casein seemed to be degraded at a higher rate. The densitometric readings (Figure 15, Table 11) showed that after 1 month of ripening about 82% of β-casein was intact; this percentage was reduced to 58.5% after 6 months and to only 43% after 9 months. Since Beemase is a blend of Actinomucor pusillus protease and bovine pepsin, its action was similar to that of Emporase.

Figures 16 and 17 represent patterns and the densitometric scans of experimental cheese made with Econozyme. The pattern depicts degradation of casein similar to that of cheese made with rennet. α_{S1}-Casein was extensively degraded; β-casein was practically intact up to 6 months of ripening. The shape of the densitograms was similar for these two cheeses especially in the region of α_{S1}-casein. According to Phelan (1973), 50/50 blends of porcine pepsin with bovine pepsin or with rennet give cheese with electrophoretic patterns indistinguishable from those given by cheese made with rennet. Because Econozyme is a blend of calf rennet and porcine pepsin it was expected for it to have lower proteolytic activity. The densitometric readings supported the idea that a greater proportion of the casein fractions remained intact compared to the cheese made with rennet. However, the

Figure 15. Densitometric scanning patterns of electrophoresis gels of experimental American Cheddar-type cheese made with Beemase, after 0, 1, 3, 6, and 9 months of ripening.

Figure 16. DISC-PAGE (9% T) patterns and facsimilies experimental American Cheddar-type cheese made with Econozyme, after 0 time of ripening (1-A, 1-B), 1 month (2-A, 2-B), 3 months (3-A, 3-B), 6 months (4-A, 4-B), and 9 months (5-A, 5-B).


ECONOZIME
B-
+
5
4
3
2
1
2
4
5
6
7
8

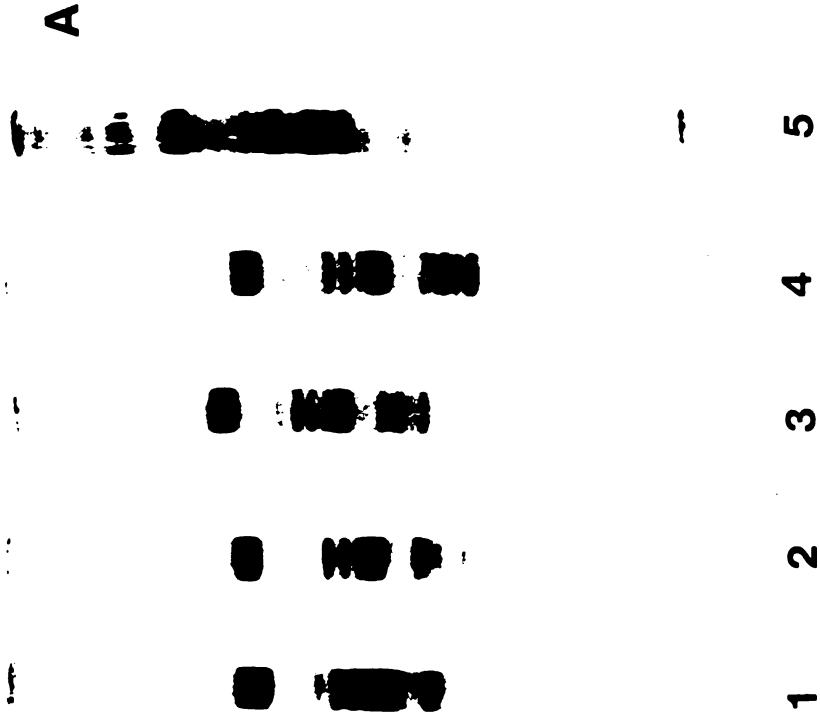

A
5
4
3
2
1

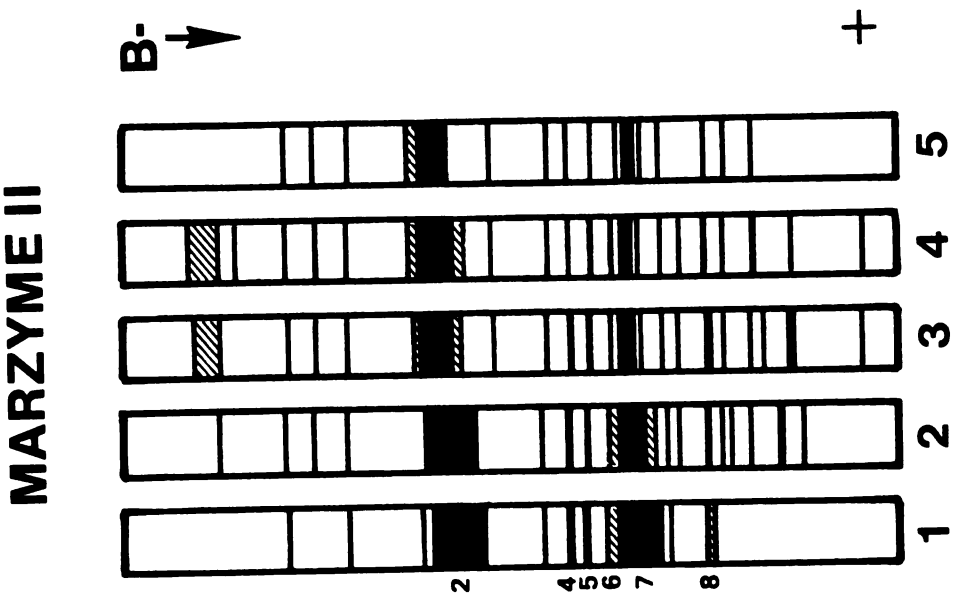
Figure 17. Densitometric scanning patterns of electrophoresis gels of experimental American Cheddar-type cheese made with Econozyme, after 0, 1, 3, 6, and 9 months of ripening.

difference was very small.

Figures 18 and 19 represent patterns and the densitometric scans of experimental cheese made with Marzyme II. The degradation in the area of α_{S1}-casein seemed similar in pattern to cheese made with the other Actinomucor preparations, i.e. Beemase and Emporase, and not to cheese made with rennet and Econozyme. However, β-casein was degraded to a lesser extent than in the case of the cheese made with Emporase and Beemase. After 9 months of ripening about 50% was still intact. These findings were in conflict to those of Vanderpoorten and Weckx (1972) who found that Actinomucor miehei preparation degraded β-casein more intensively than Actinomucor pusillus preparations. More recently El-Shibiny et al. (1976) found that the activity of Actinomucor miehei protease on β-casein was much less pronounced than that of Actinomucor pusillus preparation. In a subsequent study, El-Shibiny et al. (1977) showed that the rate of activity of Actinomucor miehei protease was greater than that of Actinomucor pusillus protease toward α_{S1}-casein.

Figures 20 and 21 depict patterns and densitometric scans of cheese made with Bovin. The pattern of breakdown of α_{S1}-casein was similar to that of cheese made with calf rennet and Econozyme. About 12% α_{S1}-casein remained intact after 9 months of ripening. O'Leary and Fox (1973) also found similar rates of degradation of casein fractions by

Figure 18. DISC-PAGE (9% T) patterns and facsimilies of experimental American Cheddar-type cheese made with Marzyme II, after 0 time of ripening (1-A, 1-B), 1 month (2-A, 2-B), 3 months (3-A, 3-B), 6 months (4-A, 4-B), and 9 months (5-A, 5-B).


MARZYME II
B-
+
5
4
3
2
1
2
4
5
6
7
8

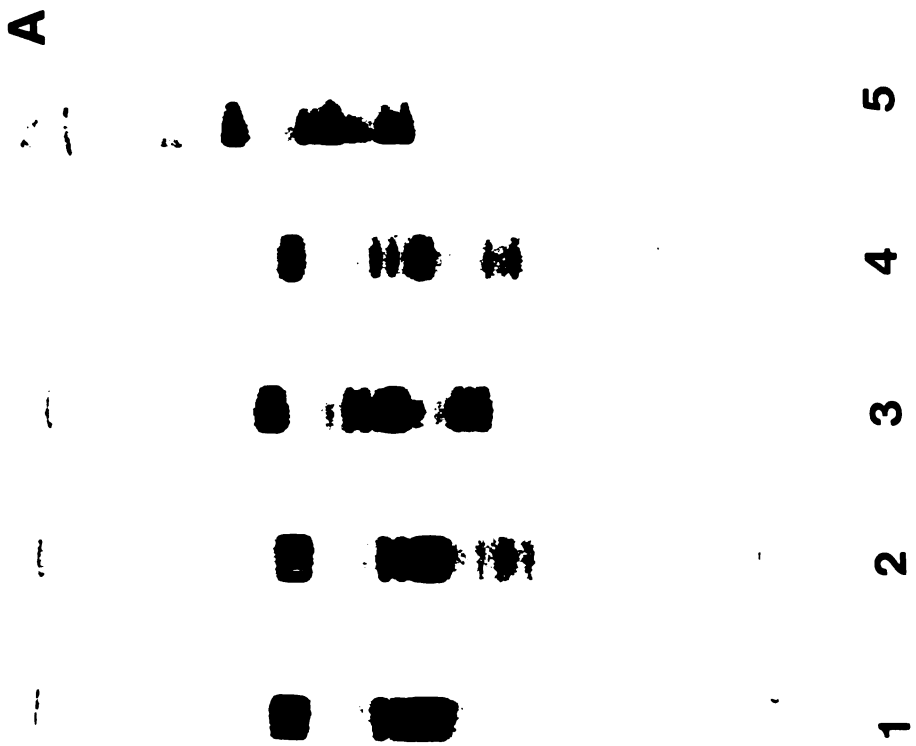

A
5
4
3
2
1

Figure 19. Densitometric scanning patterns of electrophoresis gels of experimental American Cheddar-type cheese made with Marzyme II, after 0, 1, 3, 6, and 9 months of ripening.

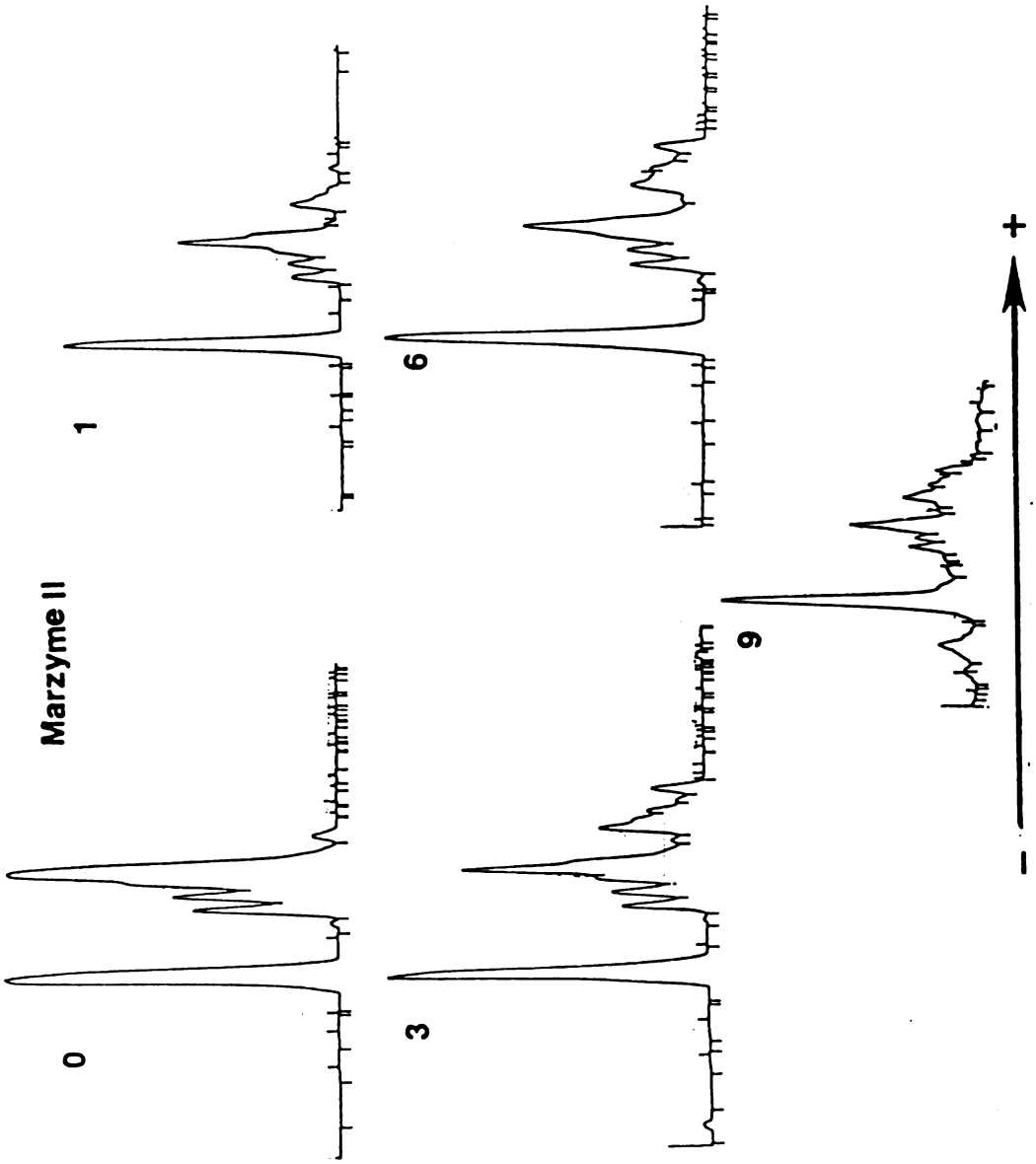
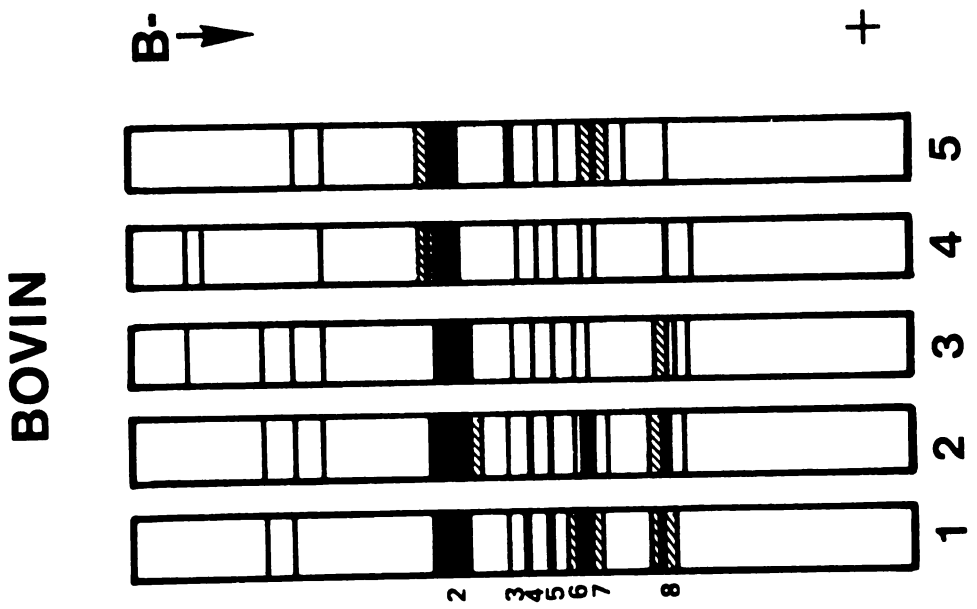

Marzyme II
0
1
3
6
9
+
-

Figure 20. DISC-PAGE (9% T) patterns and facsimilies of experimental American Cheddar-type cheese made with bovin, after 0 time of ripening (1-A, 1-B), 1 month (2-A, 2-B), 3 months (3-A, 3-B), 6 months (4-A, 4-B), and 9 months (5-A, 5-B).


B-
+
BOVIN
5
4
3
2
1
2 3 4 5 6 7 8

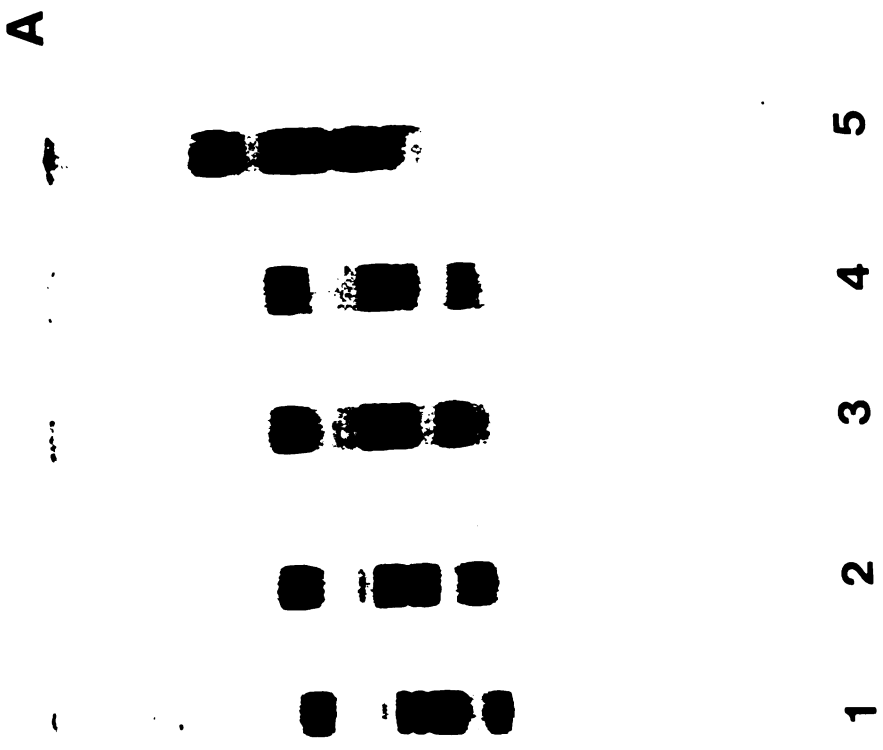

A
5
4
3
2
1

Figure 21. Densitometric scanning patterns of electrophoresis gels of experimental American Cheddar-type cheese made with Bovin, after 0, 1, 3, 6, and 9 months of ripening.

bovine pepsin and by calf rennet.

In all the six cheeses the δ region showed three distinguishable zones which may be products of degradation of β-casein by the action of a native milk protease. Creamer (1975) proposed that β-casein was slowly degraded by a milk protease in ripened cheese to give δ-, TS-, and R-caseins. The rate of degradation was probably influenced by moisture content and pH of cheese. However, Visser et al. (1977) reported that the total contribution of milk protease to degradation of α_{S1}- and β-casein in cheese was very low in relation to that of rennet and starter bacteria.

The zones of different intensity observed between α_S- and β-casein have been reported by Visser et al. (1977) as the degradation products of β-casein originally described by Creamer et al. (1971). These authors proposed that rennin cleaves β-casein in dilute solution to give B-I, B-II, and B-III, each with greater electrophoretic mobility than β-casein. However, in cheese, β-casein is resistant to rennin action because the environment is conducive to formation of β-casein polymers that are not readily hydrolyzed. It is important to notice that in that study, calf rennet was the sole coagulant used. The higher proteolysis of β-casein in some of the experimental cheeses could be due to the different coagulants used or to the lower salt content. Phelan et al. (1973) indicated

that β-casein was highly resistant to proteolysis in Cheddar cheese, but a decrease in salt concentration could reduce its resistance.

At this point the six coagulants under study can be separated into two groups. The first group includes animal coagulants, rennet, Bovin, and Econozyme (blend of calf rennet and porcine pepsin). These coagulants degraded α_S-caseins to an intensely colored zone of higher electrophoretic mobility that was degraded in time to faster, paler zones; the rate of α_S-caseins degradation was fairly high in these three enzymes. β-Casein was quite resistant to their action. The second group were the microbial enzymes (Emporase, Beemase and Marzyme II) which degraded α_S-caseins at a slower rate yielding two regions in the densitogram. They had greater proteolytic activity towards β-casein than the first group. The rate of activity of the Actinomucor miehei preparation (Marzyme II) was less pronounced for β-casein and more pronounced for α_S-caseins.

It is important to note that this electrophoretic method has certain limitations since the gels retain only large and medium size peptides. Therefore, other methods should complement it for the determination of small peptides and amino acids. The quantification of protein breakdown by use of densitometric scanning gave fairly good results. More standardization is necessary, however,

to overcome certain problems. For example, relative mobility values were close in some cases, but in others they differed considerably. Accordingly, it was necessary to compare distances between different peaks to adjust the relative mobilities. In some cases the readings were high due to merged peaks. Also, standard errors were high in some cases. A casein standard run with each set of samples would have allowed identification of the major zones in more certain terms. In the next phase of the study (SDS-DISC-PAGE) casein standards were run every time to overcome difficulties in interpretation discussed above.

In a way, this study was simplified when genetic polymorphism in the caseins was overlooked. Most authors, working with cheese systems seem to observe similar patterns of casein degradation. Thus, it is possible that the different casein variants degrade in similar manner during cheese ripening. Furthermore, although in a single cow's milk the protein patterns may be distinct, in a sample of mixed milks the patterns are less distinct and more difficult to assess when the protein moieties are separated in polyacrylamide gels.

Sodium Dodecyl Sulfate Discontinuous Polyacrylamide Gel Electrophoresis

The binding of SDS to proteins minimizes charge differences among protein molecules. Thus, the

electrophoretic migration reflects only molecular size differences. A linear relationship is obtained between the log molecular weight (MW) of a protein and its relative mobility (RM). This is a valid technique only if the following criteria are met: (1) all polypeptides (standards and unknowns) must bind the same amount of SDS on a gram per gram basis (1.4 g SDS/g protein); 2) all polypeptides (standards and unknowns) must assume the same unique conformation in the SDS complex; 3) standards and unknowns must be subjected to the same electric field strength, medium viscosity, and polyacrylamide pore size (Nielsen and Reynolds, 1978).

The SDS technique estimates the molecular weights of proteins to within 10% of those determined by other techniques (Weber and Osborn, 1969).

In this study SDS-DISC-PAGE was used to follow changes in protein size during ripening of experimental cheese made with the different coagulants. At first, 10% total acrylamide was used. At this acrylamide concentration the proteins traveled too far down the slab resulting in poor resolution of the different zones. When percentage T was increased to 13% separation improved. It is possible that increasing T to 15% might have improved resolution, especially for peptides with MW lower than 10,000 daltons, but this was not done.

From the relative mobility of the standard proteins and their known molecular weights, the equation for the best fitting straight line was calculated. For the first slab the equation was log y = -0.063X + 5.1033; the coefficient of correlation was r = -0.988. For the second slab the equation was log y = -0.063X + 5.0996; the correlation coefficient was r = -0.988. For the third slab the equation was log y = -0.065X + 5.0988; the coefficient of correlation was r = -0.990. These coefficients of correlation indicated the existence of a strong linear relationship between the relative mobility of the proteins and the log of their molecular weight. The standard curve for slab number 3 is shown in Figure 22. The curves for slabs number 1 and 2 were almost identical.

Figure 23 represents the patterns and facsimilies of the low MW standard, whole casein, and experimental cheeses made with Bovin and with Econozyme after 0, 3, 6, and 9 months of ripening.

The untreated whole casein showed a major zone at 32,000 daltons corresponding probably to α_{S1}-casein. Another band around 30,000 probably corresponded to β-casein. These values are in agreement with those reported by Mullin and Wolfe (1974). They are higher than those reported by El-Negoumy (1980) as well as values obtained by other physical and chemical methods. According to El-Negoumy (1980), molecular weights 25 to 38% higher than those

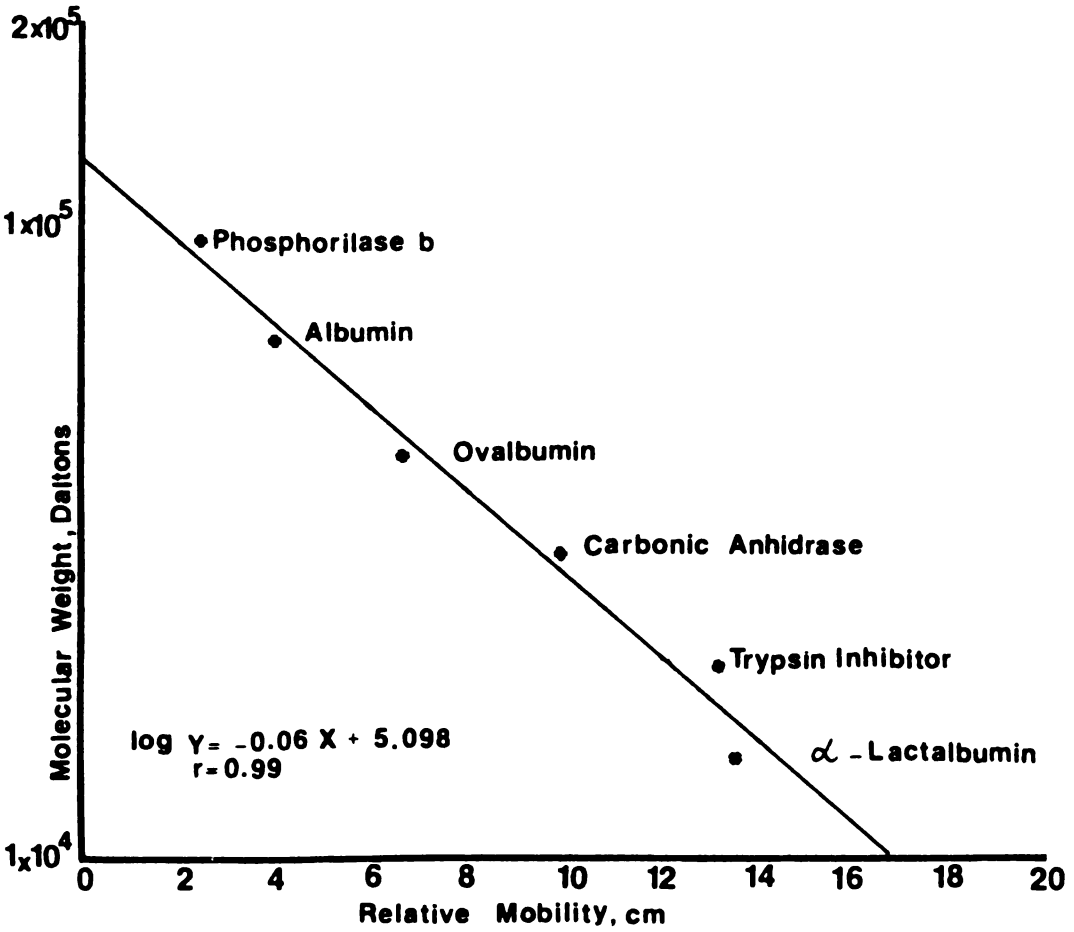


Figure 22. Standard curve for molecular weight determination in 13% SDS-PAGE.

Figure 23. SDS-DISC-PAGE (13% T) patterns and facsimilies of low molecular weight calibration kit protein (A-1, B-1); experimental American Cheddar-type cheese made with Bovin after 0 time of ripening (A-2, B-2), 3 months (A-3, B-3), 6 months (A-4, B-4), and 9 months (A-5, B-5); experimental American Cheddar-type cheese made with Econozyme after 0 time of ripening (A-6, B-6), 3 months (A-7, B-7), 6 months (A-8, B-8), and 9 months (A-9, B-9); and whole casein (A-10, B-10). The number assigned to the zones is the molecular weight in thousands of daltons.

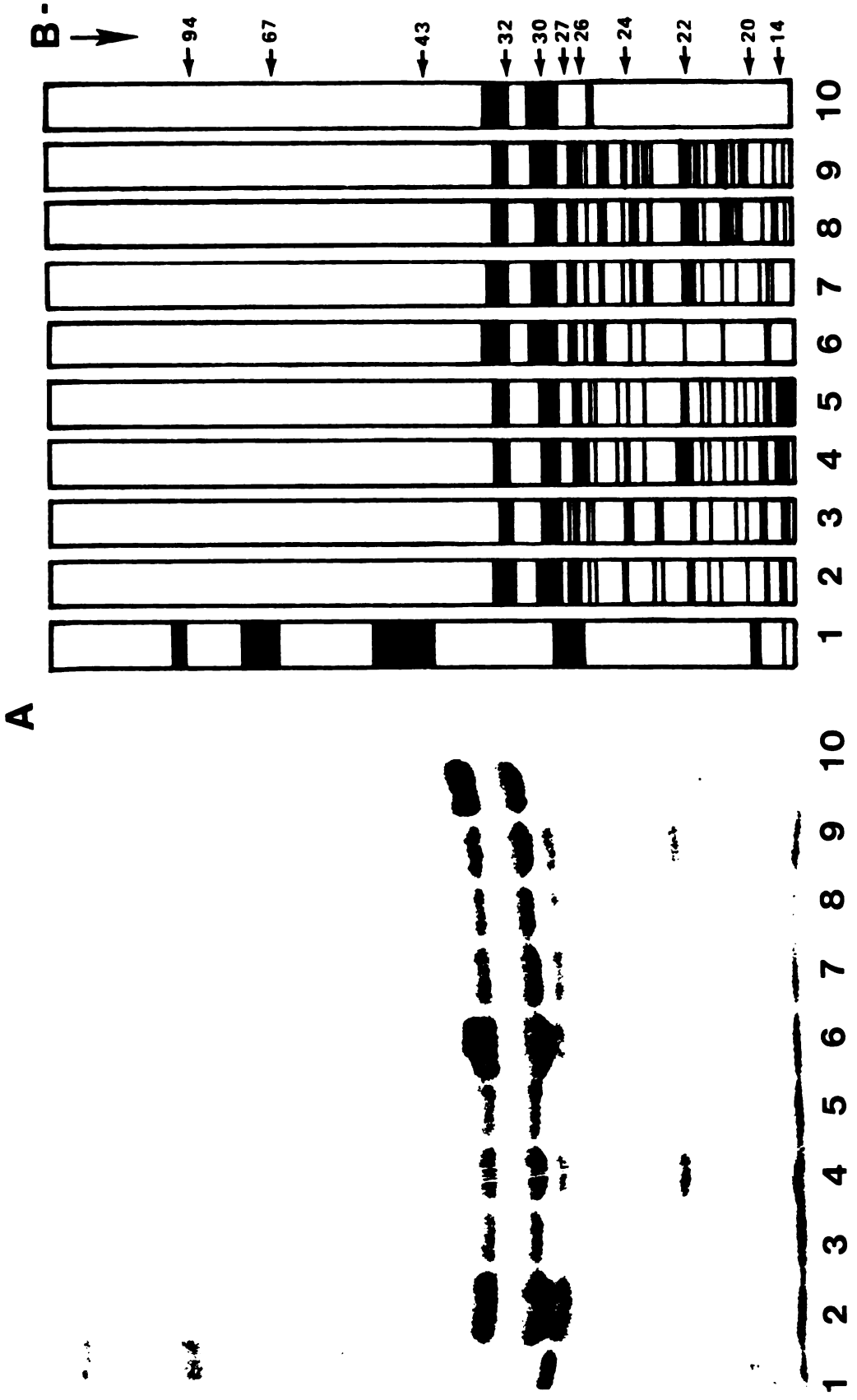
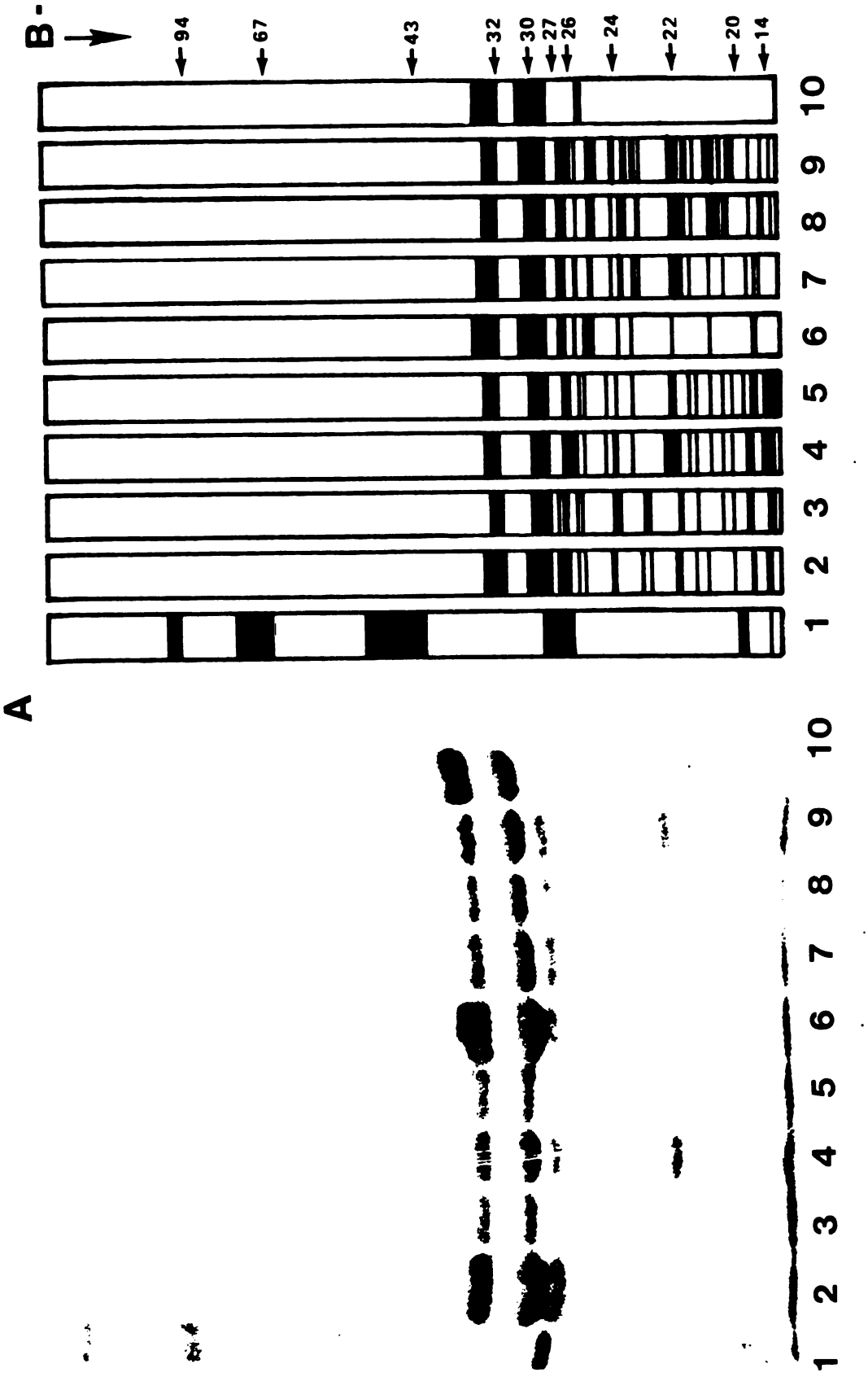

B
94
67
43
32
30
27
26
24
22
20
14
10
9
8
7
6
5
4
3
2
1
A
10
9
8
7
6
5
4
3
2
1

Figure 23. SDS-DISC-PAGE (13% T) patterns and facsimilies of low molecular weight calibration kit protein (A-1, B-1); experimental American Cheddar-type cheese made with Bovin after 0 time of ripening (A-2, B-2), 3 months (A-3, B-3), 6 months (A-4, B-4), and 9 months (A-5, B-5); experimental American Cheddar-type cheese made with Econozyme after 0 time of ripening (A-6, B-6), 3 months (A-7, B-7), 6 months (A-8, B-8), and 9 months (A-9, B-9); and whole casein (A-10, B-10). The number assigned to the zones is the molecular weight in thousands of daltons.


A
B
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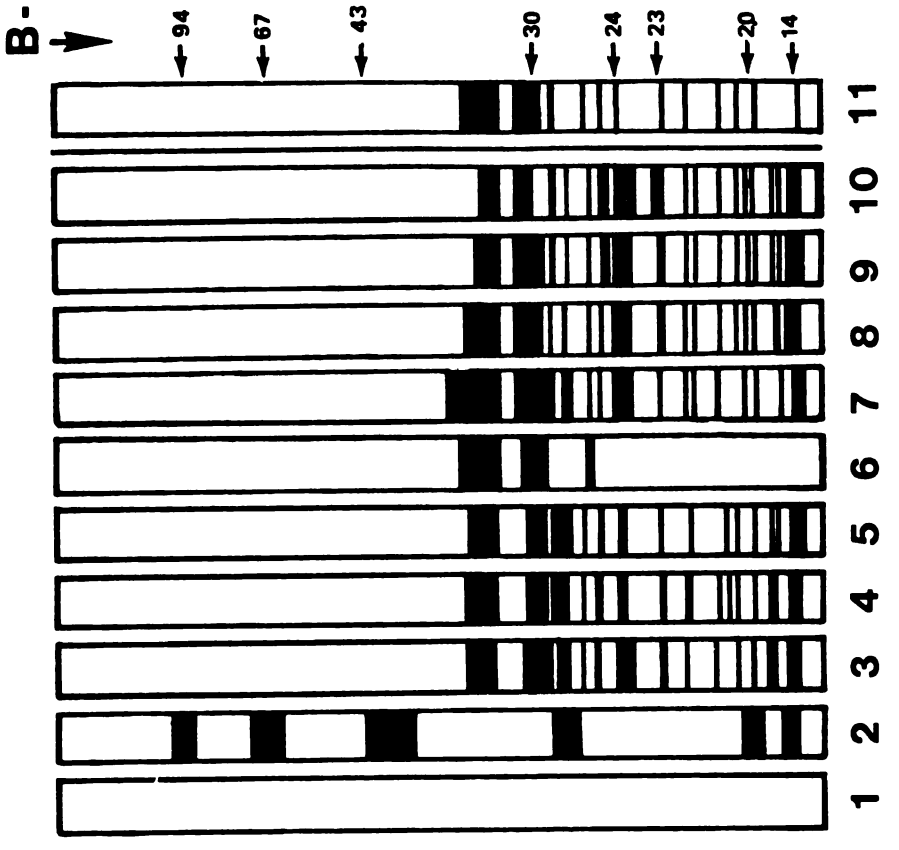
67
43
32
30
27
26
24
22
20
14
1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9 10

calculated from amino acid composition are possibly due to polymerization or aggregation through the effect of heat or SDS binding or both. Assuming that those zones correspond to α_{S1}- and β-casein, it appears that as time of ripening increased in cheese made with Bovin, α_{S1}-casein was degraded more extensively than β-casein. At 0 time the major zones were located at 32,000; 30,000; 27,000; 24,000; and 22,000 daltons. As time of ripening increased zones of lower molecular weight appeared. After 3 months the zone at 27,000 practically disappeared and a zone at approximately 26,000 daltons appeared. The zone at 24,000 intensified and so did one at 23,000 and the one at 22,000. Zones between 16,000 and 13,000 daltons increased in concentration. After 6 and 9 months of ripening the zone at 23,000 disappeared. Most of the zones at this stage of ripening had molecular weights below 18,000 and concentration of the zones around 14,000 was high.

In the case of Econozyme β-casein appeared almost identical at all times of ripening probably indicating lower proteolytic activity of this enzyme towards β-casein. Again, as time of ripening increased, the number of zones located at lower molecular weights increased, both in number and in concentration.

Figure 24 represents the patterns and facsimilies of the standard proteins, whole casein, and cheese made with

Figure 24. SDS-DISC-PAGE (13% T) patterns and facsimilies of low molecular weight calibration kit proteins (A-2, B-2); experimental American Cheddar-type cheese made with Marzyme II after 0 time of ripening (A-11, B-11), 3 months (A-3, B-3), 6 months (A-4, B-4), and 9 months (A-5, B-5); whole casein (A-6, B-6); and experimental American Cheddar-type cheese made with Emporase after 0 time of ripening (A-7, B-7), 3 months (A-8, B-8), 6 months (A-9, B-9), and 9 months (A-10, B-10). Pattern A-1 is a repetition of pattern A-11. The number assigned to the zones is the molecular weight in thousands of daltons.


B-
94
67
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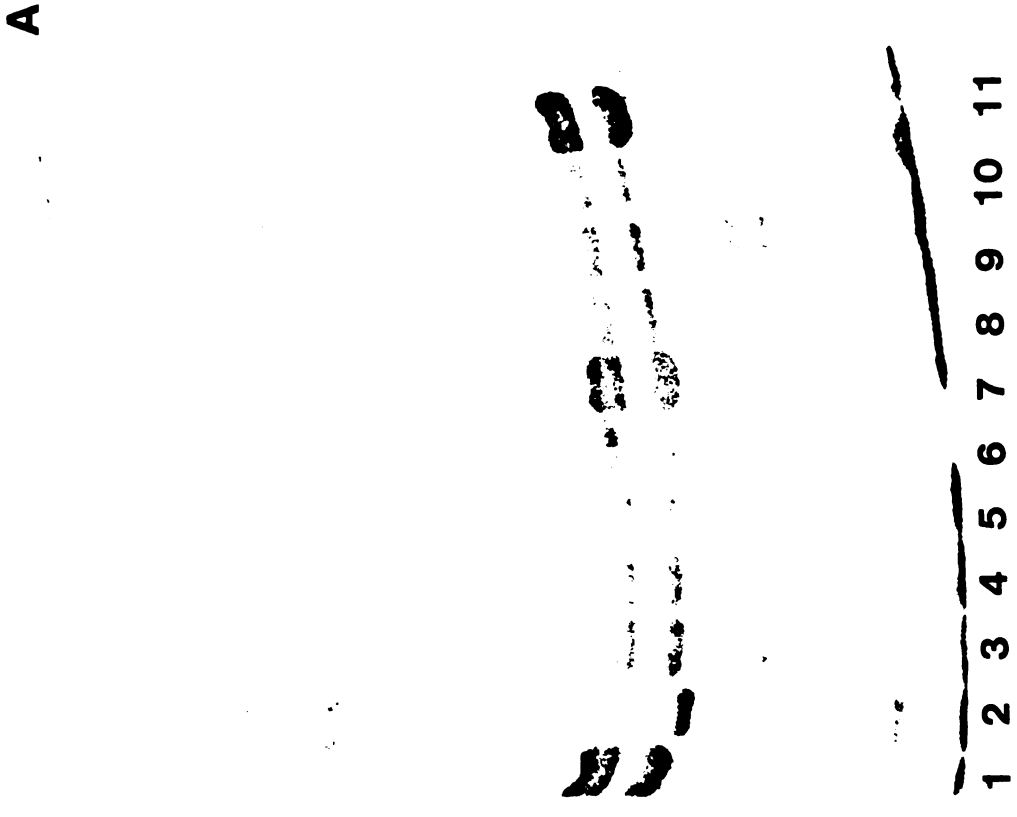
4
3
2
1


A
11
10
9
8
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5
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1

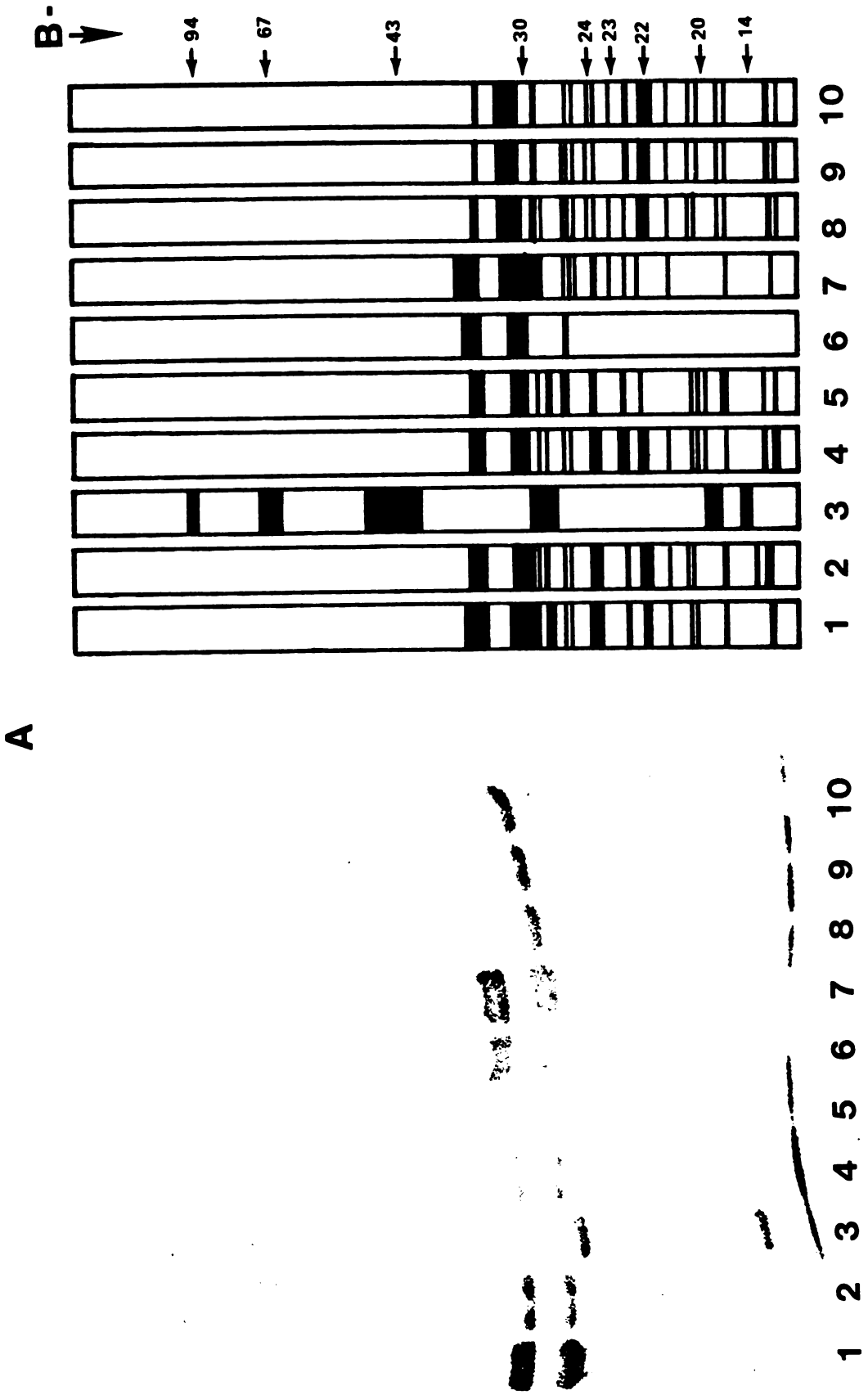
Marzyme II and Emporase.

In cheese made with Marzyme II ripening-induced reduction of β-casein was comparatively less than in cheese made with Emporase. Here, the β-casein zone was quite reduced, especially after 9 months of ripening. In both cheeses α_{S1}-casein seemed to be reduced slowly. In cheese made with Marzyme II major zones were located at 28,000; 24,000; 23,000; 16,000; and 14,000 after 3 months of ripening. After 6 and 9 months the zones at 24,000 and 23,000 were still noticeable and smaller zones around 17,000 appeared. Zones around 14,000 continued to increase in concentration.

In general, cheese made with Emporase presented a greater number of zones after 3, 6, and 9 months than cheese made with Marzyme II. Major zones were located at 24,000, 23,000, 16,000, and 15,000. They increased in concentration as a function of time. After 9 months a zone around 14,000 was noticeable.

Figure 25 depicts the patterns and facsimilies of the standard proteins, whole casein, and cheese made with Beemase and with calf rennet. α_{S1}-casein seemed to be degraded at a greater rate and extent in cheese made with rennet than in cheese made with Beemase. The contrary seemed to occur with β-casein which was degraded more in cheese made with Beemase.

Figure 25. SDS-DISC-PAGE (13%) patterns and facsimilies of low molecular weight calibration kit proteins (A-3, B-3); experimental American Cheddar-type cheese made with Beemase after 0 time of ripening (A-1, B-1), 3 months (A-2, B-2), 6 months (A-4, B-4), and 9 months (A-5, B-5); whole casein (A-6, B-6); and experimental American Cheddar-type cheese made with calf rennet after 0 time of ripening (A-7, B-7), 3 months (A-8, B-8), 6 months (A-9, B-9), and 9 months (A-10, B-10). The number assigned to the zones is the molecular weight in thousands of daltons.


B-
94
67
43
30
24
23
22
20
14
10
9
8
7
6
5
4
3
2
1
A
1 2 3 4 5 6 7 8 9 10

In general, the information gained from the SDS-PAGE study was limited by: 1) inability to distinguish the peptides that were products of degradation of α_{S1}-casein from those derived from β-casein; 2) lack of data to make inferences on peptides smaller than 14,000 daltons. Further work is suggested to supplement the information reported here. One may study the degradation of the individual casein fractions by the different coagulants and relate them to degradation in cheese. Also the use of a smaller pore size gel may resolve peptides in the lower molecular weight range.

Even so, these findings support the results obtained with the DISC-PAGE. Relatively larger number of degradation products of the caseins were observed in the cheese made with Beemase (Actinomucor pusillus and bovine pepsin blend) and with Emporase (Actinomucor pusillus preparation). The rate of degradation of α_{S1}-casein seemed to be greater for the cheese made with rennet, followed by Econozyme and Bovin. The rate was slower for the cheeses made with Marzyme II, Beemase and Emporase. The degradation of β-casein was almost unnoticeable in cheese made with rennet and Econozyme throughout ripening while it was evident in cheese made with the microbial enzymes, especially with Emporase. Works of Kosikowski et al. (1969) and Vanderpoorten et al. (1972) also indicated slightly higher rate of degradation of α_{S1}-casein for calf rennet than for

microbial enzymes and the opposite for β-casein.

The data therefore suggest that α_S-caseins and β-casein and their larger degradation products are first degraded into medium sized peptides, and as the ripening continues they are degraded further.

Organoleptic Evaluation

Collectively over 100 volatile and non-volatile potential flavor components have been identified in cheese (Kristoffersen, 1973). However, pure compound substitution in flavor experiments has not identified a single compound as the basic cause of typical flavor. Kosikowski and Mocquot (Kosikowski, 1978) proposed the component balance theory to explain how typical flavor originates in a ripened cheese: "(1) typical cheese flavor, particularly in Cheddar and other hard rennet cheeses, apparently does not depend upon a key component, but originates from a variety of substances resulting from protein fat and lactose, (2) in the individual state, these compounds have flavors other than cheese flavor. Collectively, when in certain critical balance, they result in typical cheese flavor, (3) certain individual components in this balance are more important than others. For example, some fatty acids, amino acids, amines, peptides and ketones together may develop a flavor resembling cheese but not fully. Other compounds such as esters, secondary alcohols, and

acids exert some effect on typical flavor. In fact, even the bland flavor input of neutral fat and paracasein may have importance." Ohren and Tuckey (1969) demonstrated that the lipid fraction of Cheddar-type cheese contributes more to development of flavor than any other component (milk proteins and lactose) by making cheese from skim-milk. The fat acts as a solvent for many of the flavor components and it is known to modify flavor properties of many compounds. In addition, the fat serves as a precursor for a variety of compounds: lactones, methyl ketones, esters, alcohols, and fatty acids (Day, 1967).

Proteins intervene in the flavor of the cheese through the peptides and amino acids produced; they provide much of the physical structure, body, and texture properties. In view of the involvement of proteins in flavor of cheese, the next phase of this work was to relate proteolysis during ripening to organoleptic quality of cheese.

The effect of the different coagulants on flavor, body and texture characteristics of cheese was assessed by organoleptic evaluation of the product after 3, 6, and 9 months of ripening. A panel of three trained judges evaluated the cheese for flavor and for bitterness using an intensity scale. Data obtained were submitted to statistical analyses. In addition, the panel analyzed the cheese for flavor, body and texture using the ADSA score cards. Since these cards were not originally

developed for research, their use for this purpose has been criticized (O'Mahony, 1979; Sidel, Stone, and Bloomquist, 1981). Since the ADSA procedure still serves as a guide to maintenance and quality improvement of dairy products (Bodyfelt, 1981) I considered it to be a good complement to the other analyses.

Average cheese flavor scores and average bitterness scores for experimental cheeses are presented in Table 12 and 13, respectively. The flavor scores, and body and texture scores using the ADSA official score cards are shown in Tables 14 and 15.

On the intensity scale for flavor, scores given were significantly different among the judges at all times of ripening. Therefore, the human factor became more important and the objectivity of the test could be questioned. There was no significant difference in flavor intensity due to coagulant used or due to judges coagulant interaction at any time.

After 3 months of ripening cheese made with Emporase and Marzyme II had the highest scores for cheese flavor. In the case of Emporase it may be related to its high proteolytic activity previously demonstrated. Green et al. (1975) working with Cheddar cheese made with blends of Actinomucor pusillus protease and porcine pepsin, found that the highest level of Actinomucor pusillus protease in the blend hastened the development of cheese flavor.

Table 12. Average cheese flavor scores for American Cheddar-type cheese after 3, 6, and 9 months of ripening.

Treatment	Number or Trials	Time of Ripening						Overall Mean
		3[1]		6[2]		9[3]		
		$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE	
Rennet	4	2.42	.15[a]	3.33	.31[a]	3.33	.33[a]	3.03
Emporase	4	2.67	.19[a]	3.33	.31[a]	3.58	.36[a]	3.19
Beemase	4	2.58	.15[a]	3.50	.34[a]	3.33	.28[a]	3.14
Econozyme	4	2.58	.15[a]	3.58	.26[a]	3.83	.24[a]	3.33
Marzyme II	4	2.67	.19[a]	3.17	.34[a]	3.50	.36[a]	3.11
Bovin	4	2.50	.26[a]	3.50	.29[a]	3.17	.27[a]	3.06

[a]Means within a column not followed by a common letter differ ($P<.05$).

[1]Treatment and interaction were not significantly different. Judges were significantly different ($P<.01$).

[2]Treatment and interaction were not significantly different. Judges were significantly different ($P<.01$).

[3]Treatment and interaction were not significantly different. Judges were significantly different ($P<.01$).

Table 13. Average bitterness scores for American Cheddar-type cheese after 3, 6, and 9 months of ripening.

Treatment	Number of Trials	Time of Ripening (mo)						Overall Mean
		3[1]		6[2]		9[3]		
		$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE	
Rennet	4	1.92	.23[a]	3.08	.53[a]	3.08	.50[a]	2.69
Emporase	4	1.42	.15[a]	2.33	.33[a,b]	1.67	.22[b,c]	1.81
Beemase	4	2.83	.17[b]	2.92	.34[a]	3.33	.36[a]	3.03
Econozyme	4	1.25	.13[a]	1.83	.21[a,b]	2.08	.26[a,c]	1.72
Marzyme II	4	1.25	.18[a]	1.25	.13[b]	1.33	.19[b,c]	1.28
Bovin	4	1.92	.23[a]	2.67	.33[a]	3.08	.36[a]	2.56

[a,b,c]Means within a column not followed by a common letter differ ($P<.05$).

[1]Treatment was significantly different ($P<.01$). Judges and interaction were not significant.

[2]Treatment and judges were significantly different ($P<.01$). Interaction was not significant.

[3]Treatment and judges were significantly different ($P<.01$). Interaction was not significant.

Table 14. Comparison of average flavor scores in American Cheddar-type cheese after 3, 6, and 9 months of ripening, using the ADSA official score card.

Treatment	Average flavor score*	Remarks
		3 months
Rennet	6.29	bitter (9), flat (8), acid (5)
Emporase	7.29	flat (7), acid (5), bitter (2)
Beemase	6.17	bitter (11), flat (6), acid (5)
Econozyme	7.33	flat (9), sulfide (3), acid (2)
Marzyme II	7.75	flat (10), acid (2)
Bovin	6.92	flat (6), acid (2), bitter (2)
		6 months
Rennet	6.75	bitter (7), acid (6)
Emporase	7.33	bitter (6), acid (5), flat (2)
Beemase	6.92	bitter (8), acid (4), flat (2)
Econozyme	8.00	flat (4), acid (4), bitter (2), sulfide (2)
Marzyme II	7.75	flat (5), acid (5)
Bovin	6.92	bitter (7), acid (5), sulfide (2)
		9 months
Rennet	6.75	bitter (7), acid (5), flat (3)
Emporase	7.33	acid (7), flat (4), bitter (3)
Beemase	6.63	bitter (12), acid (5), flat (3)
Econozyme	7.67	acid (5), flat (4), bitter (3), sulfide (3)
Marzyme II	7.21	flat (6), acid (3)
Bovin	6.75	bitter (8), acid (4), flat (2)

*The flavor score represent averages of 12 observations. Perfect score=10 points. Values between brackets represent the frequence of the remark.

Table 15. Comparison of average body and texture scores in American Cheddar-type cheese after 3, 6, and 9 months of ripening using the ADSA fficial core ard.

Treatment	Average body and texture score*	Remarks
		3 months
Rennet	3.38	short (10), corky (5), open (3), crumbly (2), mealy (2)
Emporase	3.33	short (9), open (7), corky (3), pasty (2)
Beemase	3.17	short (8), open (5), corky (5), mealy (3), crumbly (2), curdy (2)
Econozyme	3.83	short (5), open (3)
Marzyme II	3.58	short (6), curdy (5), corky (4), open (2)
Bovin	3.92	short (7), open (3), corky (2)
		6 months
Rennet	3.25	short (9), open (5), mealy (3), pasty (3), corky (2), curdy (2), crumbly (2)
Emporase	3.38	short (8), open (6), mealy (4), corky (2), curdy (2), crumbly (2), pasty (2), weak (1)
Beemase	3.42	short (8), open (5), mealy (3), crumbly (3), corky (2)
Econozyme	4.08	short (7), open (5), mealy (2)
Marzyme II	3.83	short (7), curdy (4)
Bovin	3.96	short (8), open (4)
		9 months
Rennet	3.29	short (10), crumbly (6), open (4), mealy (2)

Table 15. (cont'd.)

Emporase	3.50	short (12), open (6), crumbly (5), mealy (3)
Beemase	3.29	short (11), crumbly (6), open (4), mealy (3)
Econozyme	4.08	short (5), open (5), crumbly (2), corky (2)
Marzyme II	3.79	short (7), open (4), crumbly (3), mealy (1)
Bovin	3.83	short (9), open (3), crumbly (2), corky (2)

*The body and texture scores represent the average of 12 observations. Perfect score = 5 points. Values between brackets represent frequency of the remarks.

Beemase's high score may also be due to this reason. The lowest scores were those of Bovin and rennet. As expected, after 6 months flavor intensity increased for all cheeses. The score for the cheese made with Econozyme was slightly higher followed by Beemase and Bovin with the same score, Emporase and rennet with the same score and Marzyme II with the lowest score. After 9 months, Econozyme continued to have the highest score. Again, these differences in score among the cheeses made with the different coagulants were not statistically significant.

The bitterness evaluation on the intensity scale indicated that after 3 months of ripening cheese made with Beemase was the only one with significant bitterness. After 6 months cheese made with rennet and Beemase had the highest score for bitterness followed by the cheese made with Bovin and Emporase. The lowest scores were those of Econozyme and Marzyme II. The latter was significantly lower than for rennet and Beemase. After 9 months of ripening cheeses made with Beemase, rennet, and Bovin were significantly more bitter than cheeses made with Emporase and Marzyme II but not significantly different from cheese made with Econozyme.

When the ADSA forms were used the flavor scores were more consistent among judges. This was probably due to a greater familiarity of the judges with this form of evaluation. After 3 months all the cheeses were considered

flat at least 50% of the time. This was expected given the short time of ripening. Cheeses made with Emporase and Beemase were considered flat less often, especially when compared to the cheeses made with Econozyme and Marzyme II. Again, this may indicate that the level of proteolysis is important for rapid flavor development.

Marzyme II and Emporase had the highest scores after 3 months. These results agree with those from the intensity scale. In the case of Marzyme II it is likely that the highest score is due to the less number of defects in this cheese. In the ADSA score card, the defects are considered and subtracted from the overall grade. In the intensity scale the judges were only asked to identify the degree of intensity of cheese flavor in each sample. That is probably the reason for Beemase's low score in the ADSA form compared to the intensity scale. The results obtained in the intensity scale for bitterness also suggest that cheese made with Beemase was especially bitter throughout ripening.

After 6 and 9 months cheese made with Econozyme had the highest score followed by Marzyme II and Emporase. Beemase and rennet had the lowest scores. The low scores for the cheese made with rennet in both intensity scale and ADSA forms are hard to explain since rennet is considered the best coagulant for cheese manufacture. One possible explanation is the low cheese pH at all times of

ripening that may have enhanced the levels of proteolytic activity in this cheese. Correlation coefficient for pH of cheese and bitterness was .89. This was also true for cheese made with Beemase which had considerably lower scores than cheese made with Emporase even though both are Actinomucor pusillus preparations.

Acid flavor, bitterness, and lack of true cheese flavor can be caused by excessive acid development during the cheese manufacture. As mentioned earlier in this discussion, the excessive acidity in experimental cheeses may have been produced by the use of excessive starter. It could also be due to too much moisture in the cheese. Other manufacturing variables causing excessive acidity in cheese are: prolonged milk ripening period, dipping the curd too late, and too rapid acid development before the curd is firm and at milling stage (VanSlyke, 1979).

For body and texture cheese made with Econozyme had the best scores. Bovin had also high scores, while Beemase, rennet and Emporase had the lowest ones.

The most frequent defect (detected more than 50% of the time) was short body. It was particularly frequent in cheeses made with rennet, Emporase and Beemase. Short body indicates a slight degree of crumbly defect; it is generally caused by excessive acid production. Cheeses made with Beemase, Emporase and rennet had the lowest pH throughout ripening. Thus, the manufacturing procedure

may have contributed to the body defects of cheese. When pH of cheese was correlated to the frequency of the short body defect in the ADSA form, the correlation coefficient was .71.

According to Lawrence and Gilles (1980) moisture in the nonfatty substance (MNFS) and salt in moisture (S/M) are the two most important compositional factors related to Cheddar cheese quality. The MNFS is an indication of the relative amounts of moisture and protein (mainly casein) in cheese. The effect of moisture is more closely related to the amount of moisture per unit of casein than with the absolute percentages of moisture in the cheese, because enzymic reactions largely take place in casein-moisture mixture. For optimum cheese quality, they suggest 55% MNFS and 4% S/M. At less than 4% S/M, cheese is likely to grade well at a young age as faster break-down of the cheese body results in a somewhat greater smoothness. However, undesirable side effects become more apparent during curing since at storage temperatures greater than 10 C, such cheeses tend to blow and develop pronounced sulphide flavor defects.

The experimental cheese had higher than recommended values for MNFS and lower than recommended value for S/M. Sulphide flavors developed only in the cheese made with Econozyme which happened to have the lowest S/M.

In closing, even though the experimental cheese had some defects the subjective analyses through 9 months indicated that all cheeses made with the different coagulants were acceptable. Bitterness was especially apparent in the ADSA form, particularly in cheese made with Beemase. This cheese in the intensity scale for bitterness had "distinct bitterness but at low level". The rest of the cheeses had "barely detectable bitterness" or "no detectable bitterness". It is probable that most defects resulted from problems in the manufacture of the cheese (rapid acid development, high moisture, low pH, low salt) and not from the coagulants used.

Assay of Clotting Activity

The coagulation time ranged from 1 to 40 minutes (Table 16). The linearity between coagulation time and inverse enzyme activity was determined in all cases. The coefficients of correlations were $r = .926$; $r = .953$; $r = .929$; $r = .943$; $r = .957$; and $r = .935$ for rennet, Emporase, Beemase, Econozyme, Marzyme II and Bovin, respectively. These values were high enough to indicate that the inverse linear relationship between coagulation time and enzyme activity was valid for use in measuring enzyme activity. A plot of the nonlinear and linear regression for rennet is presented in Figure 26. With the other enzymes almost identical curves were obtained.

Table 16. Clotting time of different milk coagulating enzymes measured by the time needed for coagulation of Berridge's substrate at 30 C.

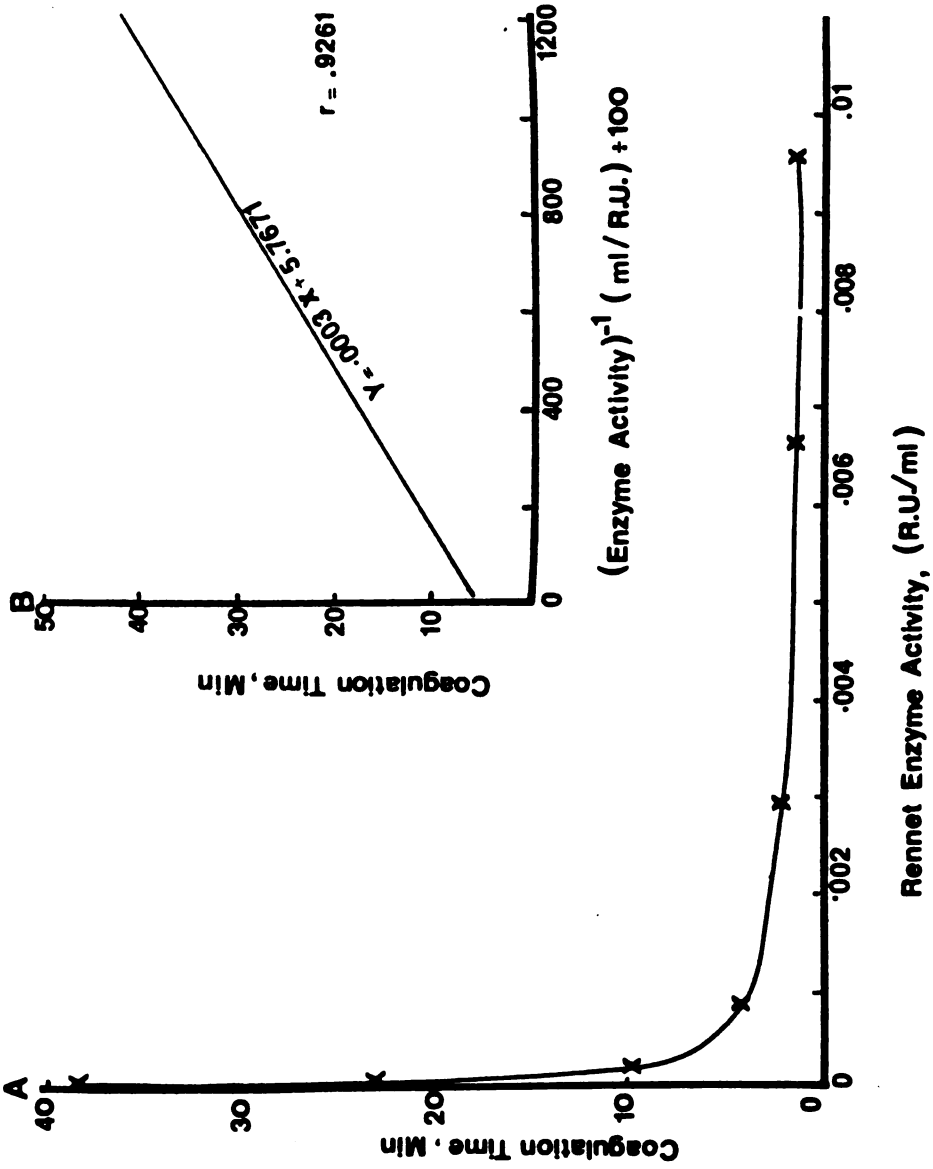
Enzyme concentration (μl/ml)[2]	Time of coagulation (min)[1]											
	Rennet		Emporase		Beemase		Econozyme		Marzyme II		Bovin	
	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE
.2	38.03	.03	31.26	1.15	36.38	.52	34.17	.00	29.97	.98	18.53	.00
.4	23.19	.10	16.33	.19	21.67	.00	19.56	.12	15.33	.00	10.33	.00
.6	16.35	.06	11.76	.14	15.74	.02	13.34	.12	10.65	.03	6.91	.00
1.0	10.00	.00	7.25	.05	9.49	.07	7.83	.10	6.28	.03	4.17	.00
2.0	4.31	.03	3.75	.01	4.55	.01	3.89	.11	3.40	.01	2.07	.01
4.0	2.27	.02	2.08	.02	2.40	.01	1.99	.04	1.77	.01	1.48	.01
6.0	1.52	.00	1.52	.00	1.72	.00	1.37	.01	1.27	.04	1.02	.01
8.0	1.39	.09	1.17	.00	1.37	.03	1.07	.01	1.03	.00	.74	.01

[1] n=3

[2] μl of coagulating enzyme per ml of diluted solution

Figure 26. A. Non linear regression of coagulation time versus calf rennet activity.

B. The least squares regression line for coagulation time versus inverse calf rennet activity.


A
B
Coagulation Time, Min
Rennet Enzyme Activity, (R.U./ml)
(Enzyme Activity)$^{-1}$ (ml/R.U.) +100
Y = .0003 X + 5.7671
r = .9261

McMahon and Brown (1983) used a Formagraph to measure coagulation time defined as time required to achieve sufficient viscosity to inhibit movement of an immersed pendulum. They obtained coefficients of correlations equal to .999. Other methods used for measuring the secondary phase of milk coagulation are based on turbidity measurements at 610 nm (Kato, Ando, Mikawa and Yasui, 1979).

From the information on enzyme concentration and coagulation time the clotting activity per ml of each enzyme used was determined. Clotting activities of the coagulants were Bovin (266 R.U./ml); Marzyme II (204 R.U./ml); Econozyme (196 R.U./ml); Emporase (175 R.U./ml); rennet (160 R.U./ml) and Beemase (154 R.U./ml).

According to Sellars (1982) bovine pepsin is more sensitive than rennin to pH's above 6.6. It continues to react at pH of 6.7 but the rate of activity is reduced noticeably. Therefore coagulants containing appreciable quantity of bovine pepsin are usually standardized higher in strength than products containing calf rennet. This is to compensate for the slower rate of activity when encountering cheese milk pH above 6.6. The higher clotting activity of Bovin, when compared to the other coagulants, could be due to this reason.

Assay of Proteolytic Activity

The liberation of 12% TCA-soluble nitrogen from Berridge's substrate was taken as a measure of proteolytic activity of the different coagulants used. The results are presented in Figure 27. After 5 min rennet liberated more 12% TCA-soluble nitrogen followed by Emporase, Beemase, Bovin, Marzyme II and Econozyme. The values were very close, however, ranging between 4 and 5% of the total nitrogen. After 25 min Bovin showed the highest proteolysis on the Berridge's substrate, followed by Marzyme II, Econozyme, rennet, Emporase, and Beemase. After 60 min Bovin, Econozyme, Marzyme II and rennet were stable, while Emporase and Beemase continued to liberate 12% TCA-soluble fraction.

The determination of clotting to proteolytic activity ratio is an accepted indicator of the suitability of an enzyme for cheese manufacture. In this work, the enzyme with higher clotting activity to proteolytic action gave the best quality cheeses.

Residual Coagulant in Cheese

Using an agar-diffusion technique, the enzyme concentrations in the cheeses made with the six different coagulants were measured after cooking and overnight pressing, and after 1, 3, 6, and 9 months of ripening.

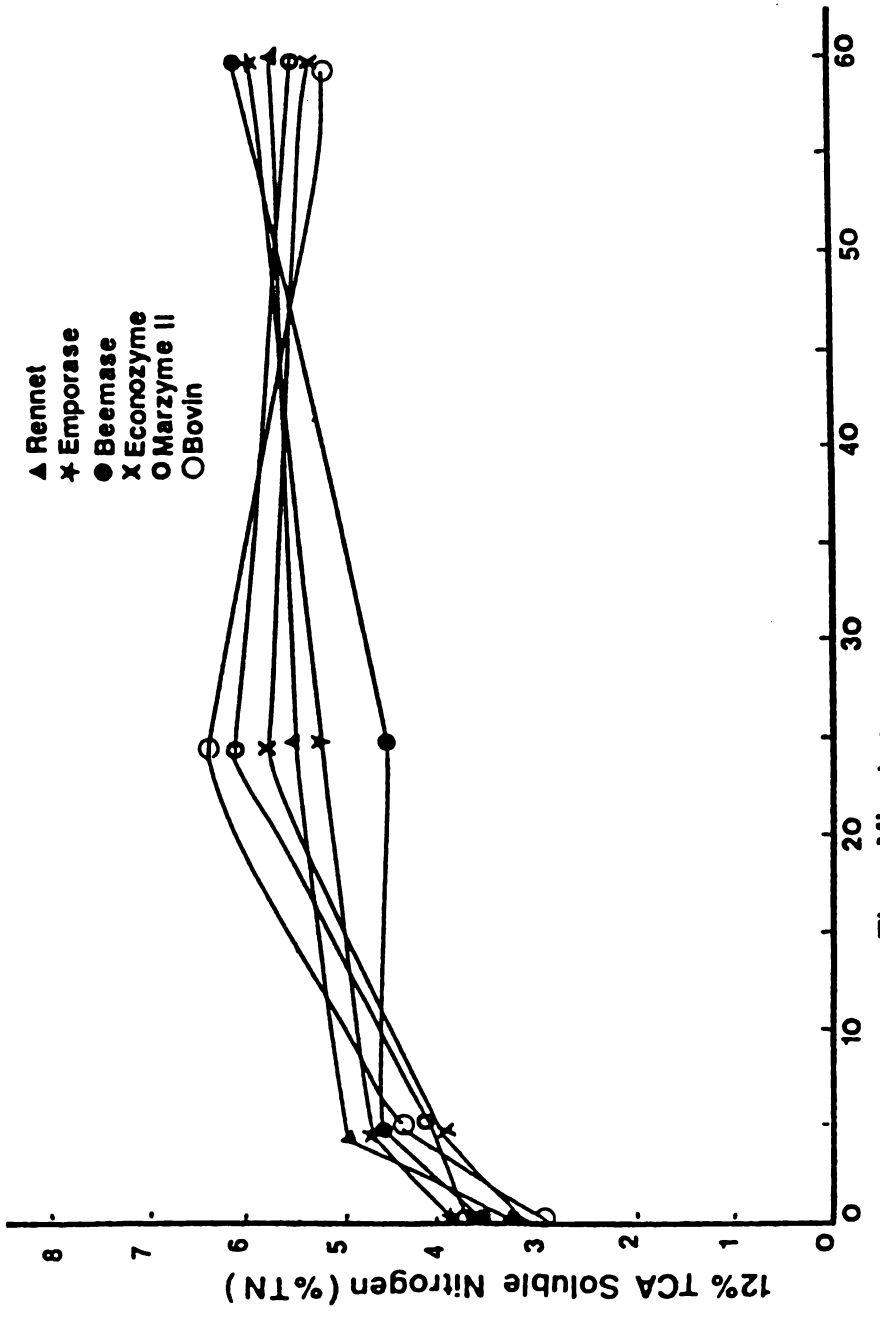


Figure 27. 12% TCA-soluble nitrogen liberated after treatment of 12% NFDM in .02N $CaCl_2$ with different milk coagulating enzymes.

From the diameter of the diffusion zones, the concentration of the enzymes in cheese was calculated using a standard curve constructed with known amounts of the enzymes (Figure 28 and 29). After overnight pressing, cheese made with rennet contained about 8.6% of the original enzyme. In time the enzyme concentration decreased slightly and at the end of 9 months about 7.23% of the enzyme could be detected. The distribution of rennet activity between curd and whey was studied by Holmes et al. (1979). They found about 6% activity left in cheese after overnight pressing.

For cheese made with the Actinomucor pusillus preparation (Emporase), after cooking, the enzyme recovered was about 6.85%; after overnight pressing, the amount recovered had decreased to 2.62% and in time it steadily decreased to about 1%. For cheese made with the Actinomucor miehei preparation, the concentration detected after cooking was about 5.4% and after overnight pressing it decreased to 4.4%; during ripening it continued to decrease up to about 3% after 9 months. Similarly, Holmes et al. (1979) found that the cheeses contained 6% and 4% of Actinomucor pusillus protease and Actinomucor miehei protease respectively, at dipping. After overnight pressing the curd contained about 3% and 1.8% of the two enzymes. In general, the data confirmed that the retention of microbial enzymes in cheese was less than that of rennet.

Figure 28. Standard curve for rennet, Emporase, Beemase, Econozyme, Marzyme II, and Bovin based on zone diffusion diameter after 20 h at 37 C. Each point is the mean of six values.

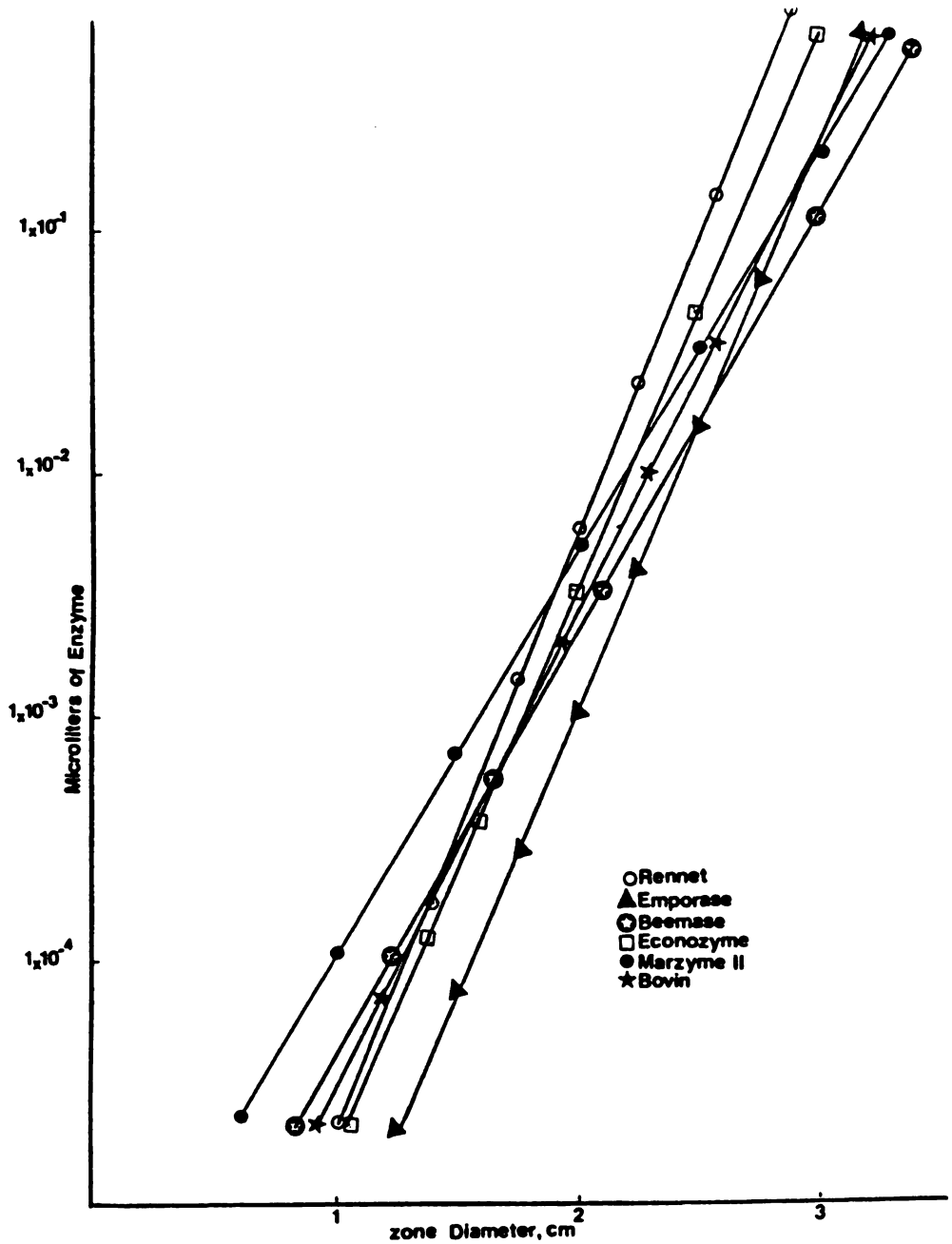
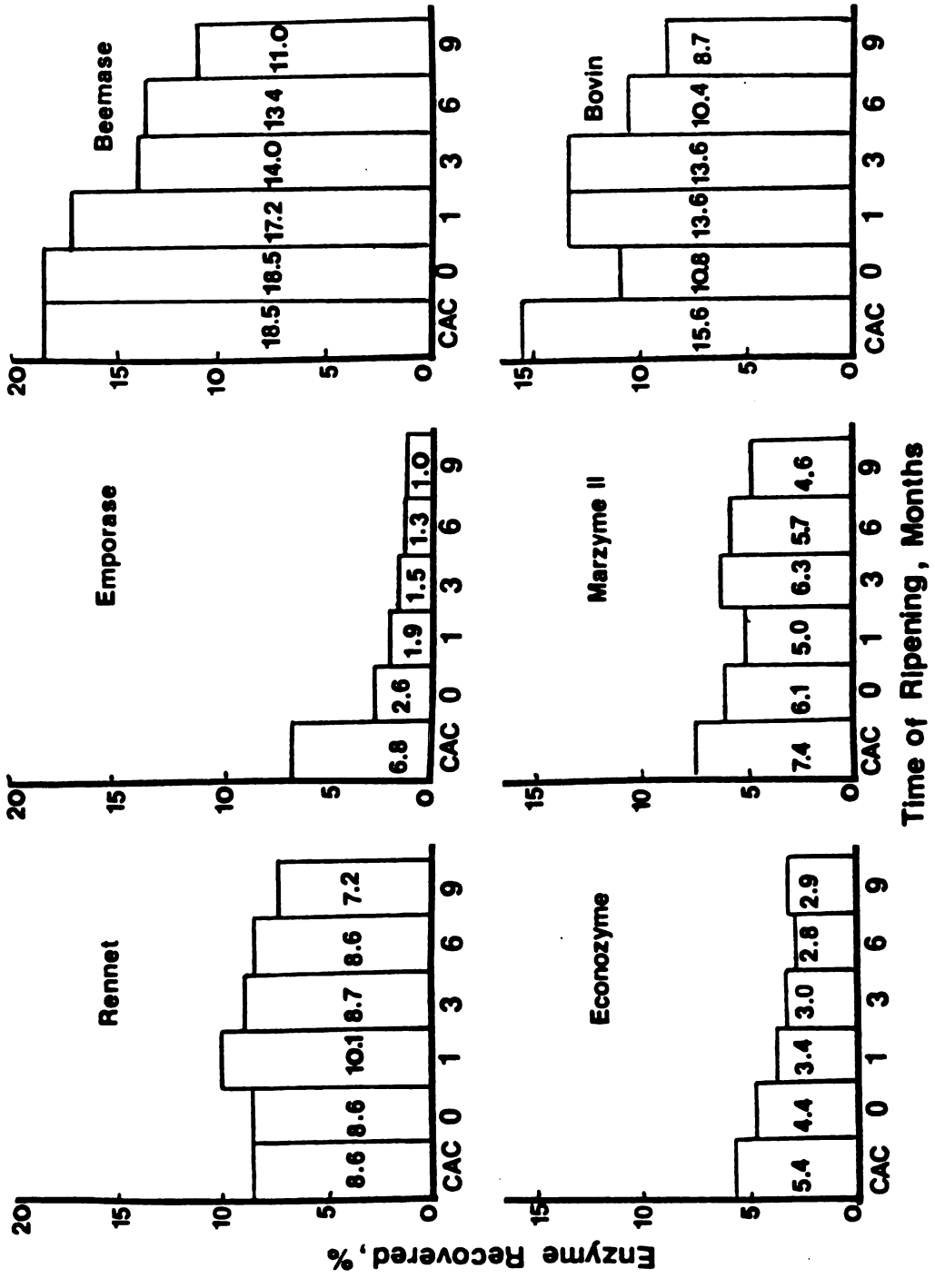

1x10⁻¹
1x10⁻²
1x10⁻³
1x10⁻⁴
Microliters of Enzyme
1
2
3
zone Diameter, cm
ORennet
AEmporase
Beemase
Econozyme
Marzyme II
Bovin

Figure 29. Percentage enzyme recovered from experimental American Cheddar-type cheese made with various coagulants after cooking (CAC), after overnight pressing (0 time), and after 1, 3, 6 and 9 months of ripening. Each number is the mean of 6 values.


Rennet
8.6
8.6
10.1
8.7
8.6
7.2
Emporase
6.8
2.6
1.9
1.5
1.3
1.0
Beemase
18.5
18.5
17.2
14.0
13.4
11.0
Econozyme
5.4
4.4
3.4
3.0
2.8
2.9
Marzyme II
7.4
6.1
5.0
6.3
5.7
4.6
Bovin
15.6
10.8
13.6
13.6
10.4
8.7
CAC 0 1 3 6 9
0 5 10 15 20
Enzyme Recovered, %
Time of Ripening, Months

When the blend of calf rennet and porcine pepsin (Econozyme) was used, 5.5% enzyme was recovered after cooking. About 4.4% was recovered after overnight pressing. With ripening, this percentage steadily decreased to about 3%. The fact that the observed retention of Econozyme was lower than rennet may be due to poor thermostability of the porcine pepsin in the blend.

When Bovin was used, a high percentage (15.6%) could be detected after cooking. After overnight pressing about 10.8% could be detected. After 1 and 3 months of ripening the amount detected was about 14%, and after 9 months it was about 9%.

When the mixture (Beemase) of bovine pepsin and Actinomucor pusillus protease was used about 18.5% could be detected after pressing, and about 11% could still be detected after 9 months of ripening. This last cheese received the lowest scores in the sensory evaluation; it is possible that the greater amount of enzyme retained in the cheese contributed furthermore to the degradation of casein in cheese. This may also explain the differences in scores for Emporase and Beemase. Little Emporase was retained in cheese throughout ripening.

From their work, Holmes and coworkers concluded that the small percentage of coagulant activity remaining in finished Cheddar cheese, particularly from microbial enzymes, suggested that the role of these enzymes in cheese

curing was minor compared to that of the enzymes of ripening microflora. In this study all cheeses were prepared in the same manner, and no significant differences in cheese flavor were observed. Only the cheese made with Beemase was particularly bitter. Therefore it is possible that at low concentration, the enzymes do not have a major effect on ripening. However, relatively high concentrations may have adverse effects.

The affinity of calf rennet for curds seems to be pH dependent (Holmes et al., 1979). However, microbial coagulants do not show a similar pH dependence. One of the reasons for the low scores in cheese made with rennet may have been the low pH of cheese that induced the coagulant retention.

Stadhouders, Hup, and Van der Waals (1977) determined the residual rennet by extraction of the cheese for 16 h with 1 M acetate buffer pH 4.9. The extract was centrifuged, dialyzed against distilled water and freeze dried. The powder was dissolved in water at 30 C and mixed with an equal volume of skim milk; the clotting time was then estimated. They found that the rennet enzymes were very stable during ripening. They reported a decrease of only .52% from 6 weeks to 4 months old Gouda cheese. In this work, rennet was also fairly stable.

Dulley (1974) extracted the residual rennet from cheese with 10% NaCl solution. The extract was combined

with 20% reconstituted milk and incubated at 37 C until appearance of the first visible coagulation. The average retention of rennet in the curd was 7%. They also concluded that while at high concentrations rennet could cause transformation of casein to peptides and amino acids, such concentrations are normally not retained in cheese. Therefore, the proteolytic effect of rennet was minimal as compared to that of the starter.

Electrophoretic Patterns of Coagulants

The electrophoretic patterns obtained for the milk coagulants are shown in Figure 30. All coagulants showed different patterns that could distinguish them between each other. However, a number of methods, most of them mentioned in the literature review, have been developed which permit more accurate identification.

Finally, Table 17 indicates the cost of the different coagulants used in this study. Since the coagulant performance in almost all cases was similar, the selection of coagulant could be based on cost alone.

Figure 30. DISC-PAGE (9% T) patterns and facsimilies of milk clotting enzymes. Standard rennet (A-1, B-1), commercial calf rennet (A-2, B-2), Emporase (A-3, B-3), Beemase (A-4, B-4), Econozyme (A-5, B-5), Marzyme II (A-6, B-6), and Bovin (A-7, B-7).

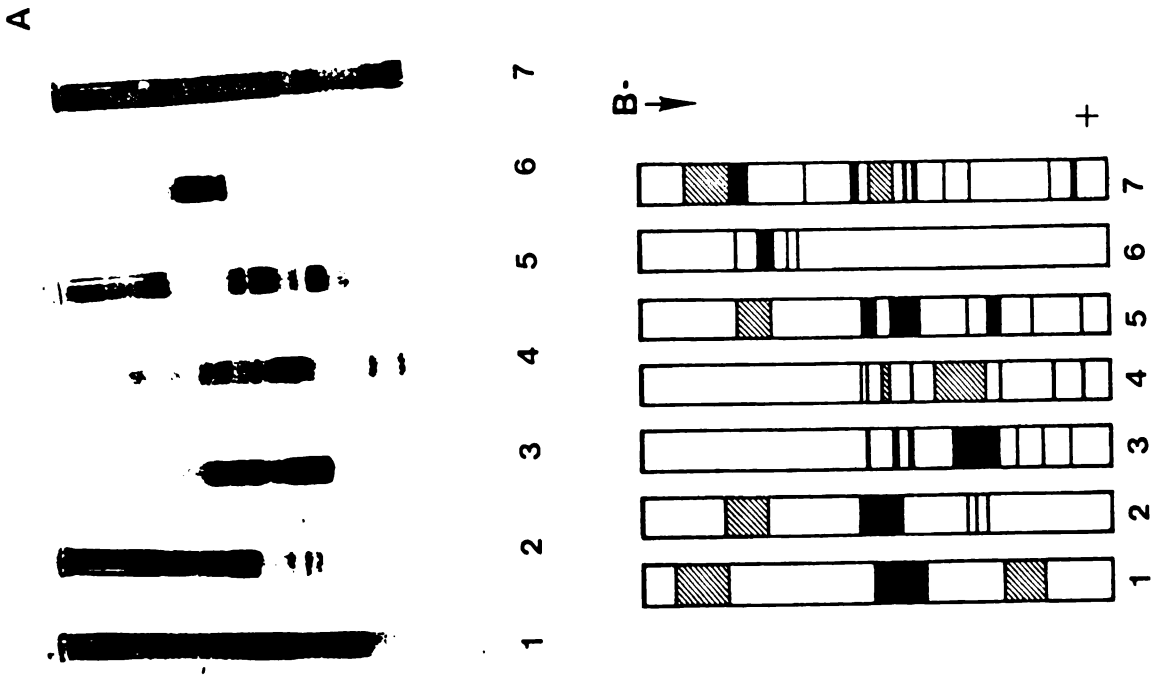

A
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Table 17. Cost of milk clotting enzymes used in this investigation[1].

Enzyme		Cost per gallon
Rennet (single strength)	Prepared by extraction of the 4th stomach of the suckling calf.	$44.00
Emporase (single strength)	Produced by the pure culture fermentation of the organisms Actinomucor pusillus lindt	$18.00
Beemase (single strength)	Blend of Actinomucor pusillus and bovine rennets	$30.40
Econozyme (double strength)	Blend of calf rennet and swine pepsin	$53.80
Marzyme II (single strength)	Produced by the pure culture fermentation of Actinomucor miehei	$18.10
Bovin (single strength)	Extract produced from adult bovine animals	$28.90

[1]Cost at Jan., 1983.

SUMMARY AND CONCLUSIONS

When American Cheddar-type cheese was made using six different coagulants, no significant difference in the composition of the cheeses was observed.

Limited pilot plant batch sizes resulted in cheese containing low salt content, slightly higher moisture, and low pH throughout ripening. Low salt and higher moisture might have accelerated the flavor and texture development of the cheese leading to more desirable conditions in discerning differences in the action of the various coagulants.

The differences in yield between the cheeses were not statistically significant. Total solids, fat, and protein content in whey which are usually used as indicators of cheese yield were not significantly different either. Yield values ranged from 9.62% for the cheese made with rennet to 9.35% for the cheese made with Beemase.

Moisture content of cheese during ripening registered no significant change with time indicating the effectiveness of the package. Relative small changes in moisture content may have been due to the opening of the packages for sample collection at different time points or due to experimental errors.

Experimental cheese pH decreased slightly at first, followed by an increase with time. The values were, however, slightly lower than normal. The rapid acid development may have caused these lower pH values. Changes in pH during cheese ripening were not significantly related to the coagulant used. Ripening time, however, produced significant pH changes.

The coagulant significantly affected the levels of pH 4.6-soluble nitrogen after 3, 6, and 9 months of ripening. The levels of 12% TCA-soluble nitrogen were significantly affected only after 9 months of ripening. Therefore, the coagulant seems to be largely responsible for the proteolysis of the caseins to large and medium size peptides, while the starter is mostly responsible for the NPN development. If pH 4.6- and 12% TCA-soluble nitrogen are used as criteria for proteolysis Emporase and Beemase, i.e., the Actinomucor pusillus preparation and the blend of Actinomucor pusillus protease and bovine pepsin, were the most proteolytic enzymes. The intermediate proteolytic activity was that of rennet and Bovin. Marzyme II (Actinomucor miehei preparation) and Econozyme (blend of calf rennet and porcine pepsin) were relatively less proteolytic.

When casein degradation was assessed by DISC-PAGE rennet, Bovin, and Econozyme degraded α_{S1}-casein to an intensely colored zone of higher electrophoretic mobility

that in time was degraded into paler, faster zones; the rate of α_{S1}-proteolysis was fairly high while β-casein was quite resistant to their attack. The microbial enzymes (Emporase, Beemase and Marzyme II) degraded α_{S1}-casein at a slower rate and gave place to two regions of peaks in the densitogram. The microbial coagulants had greater activity towards β-casein. In all 6 cheeses the δ region showed three distinguishable zones that may be δ-, TS-, and R-caseins. They were products of degradation of β-casein by alkaline milk protease. The zones between α_{S1}- and β-casein may be additional products of degradation of β-casein. The lower salt content in the cheese might have made β-casein more prone to coagulant attack.

Data of SDS-DISC-PAGE was in agreement with the DISC-PAGE results. The number of products of degradation of the caseins were larger in cheese made with the microbial enzymes. The rate of degradation of α_{S1}-casein seemed greater for cheeses made with rennet, Econozyme and Bovin but degradation of β-casein was almost unnoticeable while it was significant for the microbial enzymes.

Sensory evaluation indicated no significant differences in flavor in cheese made with the different coagulants. Cheese made with blend of calf rennet and porcine pepsin (Econozyme) got higher scores in both intensity and ADSA procedures. Cheese made with Marzyme II also had high scores. Cheeses made with Actinomucor pusillus preparation

(Emporase) and blend of Actinomucor pusillus protease and bovine pepsin (Beemase) had high scores early in ripening probably indicating a faster development of flavor in the cheese made with the more proteolytic enzymes. Cheese made with Beemase was the only one significantly bitter after 3 months of ripening. This defect was continuously detected. Common defects for all cheese samples were: bitterness, acidity and short body. They may be related to excessive acidity development during the cheese manufacture. Manufacturing conditions resulting in low salt, high acidity, high moisture, and low pH in cheese might have enhanced proteolysis but not to a level to consider the cheese unacceptable.

With the information gained on enzyme concentration and coagulation time, the highest clotting activity was observed for Bovin, followed by Marzyme II, Econozyme, Emporase, rennet, and Beemase. Measurement of proteolytic activity of the enzymes on Berridge's substrate indicated that after 60 min, Emporase and Beemase continued their proteolytic action while the other enzymes levelled off in their liberation of 12% TCA-soluble nitrogen.

The residual coagulant investigation indicated that bovine pepsin and the blend containing bovine pepsin and Actinomucor pusillus protease were retained in cheese the most. Rennet was retained more than the microbial enzymes. Econozyme, which contained calf rennet and porcine pepsin,

was retained less than pure calf rennet, probably because of the poor thermostability of the porcine pepsin.

Since all cheeses received similar sensory scores it is possible that at small concentrations the enzymes do not have a major effect on ripening. However, if concentrations were higher, they may have had adverse effects. This may have been more pronounced for Beemase cheese which displayed relatively bitter flavor.

This study showed differences in proteolysis in cheese but these differences were not necessarily adverse to the composite flavor and texture of the finished cheese. However, cheeses made with more proteolytic enzymes generally were graded lower. The contribution of other proteolytic systems (starter, milk protease, contaminants) cannot be excluded. It seems however that the coagulating enzyme liberates mostly large and medium size peptides in cheese.

The blend of Actinomucor pusillus and bovine rennet (Beemase) posed minor problems, but in general all the milk coagulants studied performed well. The results of this study demonstrated feasibility of the use of these rennet substitutes in cheese production, as long as good manufacturing practices are considered. Cost effectiveness of some of them makes them quite attractive.

APPENDIX

Appendix Table A1. List of chemicals used in this study.

Chemical	Reference Number	Company
Acetic acid Glacial	3121	Mallinckrodt
Acetone	1177	Mallinckrodt
Acrylamide monomer	161-0100	Bio-Rad
Agar noble	0142-02	Difco Laboratories
Ammonium hydroxide	2440	Mallinckrodt
Ammonium persulfate	161-0700	Bio-Rad
Bacto isoelectric casein	0145-15	Difco Laboratories
Boric acid	2549	Mallinckrodt
Bromophenol blue	332	National Aniline Div.
Calcium chloride	1-1332	Baker
Coomassie brilliant blue R-250	161-0400	Bio-Rad
Coomassie brilliant blue G-250	161-0406	Bio-Rad
Ethanol	-	Aaper Alcohol Chemical
Ethyl-ether	0844	Mallinckrodt
Ferric ammonium sulfate	1-75	Fisher
Glycerol	1-2136	Baker
Glycine	161-0718	Bio-Rad
Hydrochloric acid	2612	Mallinckrodt
Maleic acid	MX100	Matheson, Coleman and Bell
2-Mercaptoethanol	161-0710	Bio-Rad
Mercuric oxide	M-174	Fisher
Methylene blue	922	National Aniline Div.

Appendix Table A1. (cont'd.).

Methyl red	2696	Baker
Nitric acid	2704	Mallinckrodt
N,N-Methylenebisacrylamide	161-0200	Bio-Rad
N,N,N',N'-tetramethyl-ethylenediamine (TEMED)	161-0800	Bio-Rad
Perchloric acid	2766	Mallinckrodt
Petroleum ether	PX-0424	MCB
Phenolphthalein	6600	Mallinckrodt
Photo-Flo 200	-	Eastman Kodak
Potassium permanganate	7068	Mallinckrodt
Potassium sulfate	7140	Mallinckrodt
Potassium thiocianate	3326	Baker
2-Propanol	11003-5	Aldrich
Silver nitrate	S-181	Fisher
Sodium acetate	1-3470	Baker
Sodium chloride	7581	Mallinckrodt
Sodium dodecyl sulfate	161-0301	Bio-Rad
Sodium hydroxide	7708	Mallinckrodt
Sodium thiosulfate	-	Fisher
Sucrose	8360	Mallinckrodt
Sulfuric acid	2468	Mallinckrodt
Trichloroacetic acid	2928	Mallinckrodt
Tris (Hydroxi-methyl) amino methane	161-0716	Bio-Rad
Urea	8648	Mallinckrodt

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