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Protein Recovery and Coagulation Properties of Commercial and Fractionated Milk Clotting Enzymes

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PROTEIN RECOVERY AND COAGULATION PROPERTIES OF COMMERCIAL AND FRACTIONATED MILK CLOTTING ENZYMES

by

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To my parents

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Khalida A. Shaker

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ABSTRACT

Protein Recovery and Coagulation Properties of Commercial and Fractionated Milk Clotting Enzymes

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Protein recovery and coagulation properties of five commercial and fractionated milk clotting enzymes were studied. The fractionated enzymes were Sephadex G-100 fractions of the commercial enzymes. Milk clotting activity of each fraction was tested using Berridge substrate. All fractions from each preparation which had milk clotting activity as measured with the Formagraph were collected and pooled. These samples and the original enzyme preparations were used to coagulate milk. Percent of protein lost in whey was determined by Kjeldahl. Coagulation was followed using a spectrophotometer monitoring changes in apparent absorbance at 600 nm. Curd protein yields using the five original enzyme preparations were compared with each other. Also, protein lost in whey from the five original preparations were compared with those using the isolated fraction. There was a significant difference among the original enzymes in protein lost in whey. There were also

significant differences between some of the commercial enzyme preparations and their fractionated preparations. Gel filtration through Sephadex G-100 improved bovine rennet and calf rennet/porcine pepsin mixture more than the other three enzyme preparations. Calf rennet, Mucor miehei protease and modified M. miehei protease showed no significant reduction in protein lost to whey after fractionation. Protein loss using original calf rennet, bovine rennet and modified M. miehei protease were not significantly different from each other. M. miehei protease and calf rennet/porcine pepsin mixture were not significantly different from each other, but, the two groups were significantly different from each other.

There were noticeable differences in coagulation curves of the five original enzymes. Coagulation properties of commercial and fractionated enzyme were different in all five pairs.

(77 pages)

INTRODUCTION

Proteolytic enzymes are used to cause milk coagulation for manufacture of cheese. Most proteolytic enzymes obtained from bacteria, fungi, and animal organs are able to clot milk. Some are recommended as substitutes for calf rennet in cheese making, but most cause extensive digestion of cheese curd.

Until recently the enzyme used for commercial manufacture of cheese was chymosin (rennin, EC 3.4.23.4), the gastric enzyme of calf, in form of a crude extract, powder or paste called rennet. Chymosin is still the most desirable enzyme for this purpose and is the standard of evaluation for other milk clotting enzymes. But there is no longer enough chymosin available to satisfy the needs of the cheese industry and this has forced people to search for suitable and inexpensive substitutes.

Enzymes from animal organs are obtained as zymogens (proenzymes) which must be activated. Prochymosin is converted to chymosin by enzymatic proteolysis below pH 5. At room temperature prochymosin is stable in the pH range of 5.5 to 9.0 (Foltmann, 1971). Chymosin stability is affected by temperature, salt and pH. Chymosin solutions have optimum stability at pH 5.3-6.3. Cheeseman (1969) reported on the basis of sedimentation equilibrium studies, that at pH 3.1 chymosin solutions contain a monomer of molecular weight 30,000 but at pH 5.8 to 6.5 a dimer is predominant. Among animal proteases which have interested the cheese industry, porcine pepsin is most acceptable as a replacement for rennet in making a

number of varieties of cheese. It is difficult to get satisfactory milk coagulation using porcine pepsin alone. Porcine pepsin also shows high sensitivity to heat at pH's above 6.0, so it can be easily destroyed when no longer needed.

Proteolytic enzymes have been isolated from Mucor miehei, Mucor pusillus, Bacillus subtilis, Bacillus cereus and Endothia parasitica. Bacillus enzyme preparations are not suited for cheese making while the fungal derived enzymes show generally good results.

Milk clotting preparations from fungal sources (especially M. miehei and M. pusillus) have been used in large quantity by the cheese industry. These preparations have general protease activity in addition to the specific proteolysis responsibility for coagulation which is called milk clotting activity. The general proteolysis weakens cheese curd, reduces yield and produces peptides during ripening which give bitter flavor to cheese (Sternberg, 1972). M. miehei protease produces Cheddar cheese and other hard type cheeses of satisfactory quality without bitter flavor (Ernstrom and Wang, 1974). Chymosin substitute preparations can be improved by removing those fractions with high proteolytic activity and low milk clotting activity (Shaker and Brown, 1984).

The purpose of this work was to investigate the effects of commercial milk clotting preparations on protein recovery in curd and on the course of coagulation. Both commercially available enzyme preparations and preparations improved by gel fractionation were evaluated.

LITERATURE REVIEW

Enzymatic Coagulation of Milk Casein

Milk clotting is a complex process involving both enzymatic and non-enzymatic phases (Ernstrom and Wang, 1974). Factors such as temperature, pH, Ca^{+2} concentration, season of year (McDowell, et al., 1969), and type of milk clotting enzyme have a great effect on the time required for clotting of milk (Birkkgair and Thomsen, 1970; Dill and Roberts, 1959; Green, 1972; Green, 1977; Kowalchyk and Olson, 1978; and McDowell, et al., 1969). Less information is available on effects of various milk clotting time and rates of firming of the clot. Effects of concentration of $CaCl_2$ added to milk and seasonal effects were determined also by many workers.

Enzymatic coagulation of milk involves three separate but overlapping stages, enzymatic proteolysis, aggregation, and gelation. The observable change which occurs when milk is treated with a coagulating enzyme is formation of a visible clot. Chemical analysis reveals no gross change in composition (Wright, 1924). The main physiological function of chymosin is to digest milk in the acid environment of the calf's stomach. Pepsin can perform the same function in other animals and in humans. Chymosin is not present in the gastric secretion of normal human infants (Malpress, 1967). Despite the low pH's at which they function physiologically these enzymes rapidly clot fresh milk at pH 6.6 to 6.8 where their general proteolytic activities are extremely low. The enzymatic action necessary to cause milk clotting is apparently very specific.

In the early years Hammersten, (1914) and Van Slyke and Bosworth (1914) attempted to explain clotting of milk by chymosin on the basis of casein homogenity. They proposed that chymosin converts native casein into a new form called paracasein, which clots in the presence of calcium ion.

In the enzymatic or primary phase of milk clotting the enzyme attacks κ-casein to destroy its stabilizing capacity. During this stage the κ-casein is usually attacked by the protease in the region of the bond Phe_{105}-Met_{106} to yield two peptides with different properties. The macropeptide moiety (residue 109-169) is hydrophilic, soluble in water, and can diffuse away from the micelle after κ-casein splitting, whereas the para-κ-casein moiety (residues 1-105) is strongly hydrophobic and remains in the micelle.

Micelles are stable at least partly because micelles possess an overall negative charge on their surface giving an electrokinetic ζ-potential variously estimated as between 10 and 20 mV (Green and Crutchfield, 1971; Pearce, 1976; and Darling and Dickson, 1979). This surface charge provides a barrier to close approach and coagulation of micelles. The macropeptide carries an appreciable part of this negative charge and is hydrophilic, interacting well with solvent to provide a boundary layer around the micelle. Treatment of micelles with rennet reduces the ζ-potential by 5 to 7 mV (Green and Crutchfield, 1971; and Pearce, 1976), presumably by loss of the macropeptide, and also creates a much more hydrophobic micellar surface due to para-κ-casein. These charges suffice to

alter the balance of attractive and repulsive forces between micelles so that coagulation becomes possible.

Coagulation is controlled not only by interactive energy contributions but also by entropic factors (hydrophobic interactions), both of which contribute to the overall free energy charge which governs the reaction. It is probable that the dominant forces are hydrophobic, since coagulation is very dependent upon temperature and tends towards dissociation at low temperature.

In native casein micelles calcium ions bound in the calcium phosphate or to the casein also play a part in defining the charge on the micelle and thus on its subsequent activity towards aggregation. Both α_s-casein and β-casein are capable of binding calcium ions. Since this binding is temperature dependent (Dalgleish, 1980 and 1981) the variation of rennet clotting time with temperature may reflect changes in binding of calcium ions to casein and possibly also changes in the calcium phosphate content of micelles.

Chymosin attack on κ-casein appears to be somewhat dependent on the presence of intact histidine near the susceptible bond (Hill and Laing, 1965). Hill (1969) reported that histidine and serine residues appear to have catalytic effects rather than serving as enzyme binding sites. The chymosin susceptible bond in κ-casein also appears to be the principal target for non-chymosin milk clotting enzymes. Dennis and Wake (1965) reported that chymosin, pepsin and chymotrypsin all destroyed the stabilizing power of κ-casein in the same way, and gave rise to the same insoluble

products. The general proteolytic activities of chymosin, pepsin, chymotrypsin, a microbial protease (De Koning, 1967), E. parasitica protease (Larson and Whitaker, 1970) and M. pusillus protease (Yu, et al., 1968) vary, but their milk clotting activities seem to result from the same specific action on κ-casein.

Normally, aggregation of caseinate micelles begins before enzyme action is complete. Thus, the primary and secondary phases of coagulation overlap (Peters and Nelson, 1960). Berridge (1942) was able to separate the two phases by taking advantage of their different temperature coefficients. The enzyme reaction proceeds at a reduced but reasonable rate at 0°C without producing clotting. By quickly raising the temperature of chymosin-treated milk to various levels, he was able to measure clotting time as a function of temperature and determine the temperature coefficient for the non-enzymatic phase of milk clotting. The difference between temperature coefficients of the enzymatic and non-enzymatic stages of coagulation has been useful not only as a basis for estimating the time of the non-enzymatic stage at various temperatures (Berridge, 1942; and Peters and Nelson, 1960), but also as the basis for a continuous curd forming process in which cold milk was treated with rennet for an extended period, then quickly brought to a temperature where curd formed instantly (Berridge, 1961 and 1963).

Chymosin Substitutes

The rennet deficiency has necessitated a substitution by other enzymes whose proteolytic properties and coagulating activity are

similar to that of rennet (Chojnowski, et al., 1981). Rennet extracted from the abomasum of young suckled calves, has traditionally been used for coagulation of milk for cheese making. The characteristic of rennet (chymosin) which makes it so suitable for cheese manufacture is its high ratio of milk-clotting to proteolytic activity. Chymosin exhibits a high degree of specifity which results in its very limited ability to hydrolyse protein substrates (Fish, 1957; Bang-Jensen, et al., 1964).

In recent years, many studies have aimed to reduce the amount of rennet used for cheese manufacture. Such may be possible by coagulating with immobilized enzymes which could then be reused (Taylor, et al., 1976). But immobilized enzymes loose their activity and the immobilization process is expense. These enzymes lose activity because of the deposition of proteins and peptides on the immobilized protease, and the enzyme may slowly detach itself from the support (Taylor, et al., 1977; Cheryan, et al., 1975). Furthermore, immobilized enzymes are not transferred to the coagulum, thus limiting the process of proteolysis during cheese ripening.

Non-chymosin milk clotting enzymes have been used only in recent years. All proteolytic enzymes will clot milk under proper conditions (Berridge, 1954). They can be obtained from plant sources, bacteria, fungi, and animal organs. Most plant proteases have been unsatisfactory because of reduced yields of cheese, pasty bodied cheese, and bitter flavor (Lee, 1975).

Milk clotting enzymes from microbial sources have received considerable attention. These enzymes show greater proteolytic activity than calf rennet (Mickelsen and Fish, 1970 and Richardson, et al., 1967). Bacterial preparations are not used commercially because they impart off-flavor, render poor body and texture, and show excessive proteolysis (Green, 1972, Puhan, 1969 and Puhan and Steffen, 1967). Preparations from Bacillus subtilis, B. megaterium, and B. cereus produce lower yields of cheese due to continued proteolysis beyond milk coagulation by the bacterial proteases (Puhan and Irvine, 1973 and Singh, et al., 1967).

Some fungal protease preparations have been found acceptance as milk clotting enzymes. Milk clotting enzymes from Endothia parasitica, Mucor miehei and Mucor pusillus have been accepted as suitable and safe substitutes for calf rennet in the manufacture of all varieties of cheese (Kirk, 1969 and Sam, 1972).

Milk clotting enzyme from M. pusillus var. Lindt has been used as a chymosin substitute in a number of cheeses and has given satisfactory results (Richardson, et al., 1967). However, bitter flavor developed in Cheddar cheese after 14 months of ripening. M. pusillus proteases are less proteolytic than those of E. parasitica but both of them are more proteolytic than chymosin (Mickelsen and Fish, 1970). Commercial E. parasitica protease is used in manufacture of Swiss cheese of high quality. High cooking temperature (51.7-54.41°) is responsible for preventing excess proteolysis and allowing desirable quality. However, it produces Cheddar cheese and other low cooking temperature cheeses with bitter

flavor and inferior body compared with control cheese made with rennet (Sardinas, 1968).

A protease obtained from M. miehei was tested in Europe and has been approved for use in the U.S. M. miehei protease was completely satisfactory for Swiss cheese (Lee, 1975). Prins and Nielson (1970) reported Cheddar cheese manufactured with M. miehei protease to be of good quality even after extended curing. Ernstrom and Wang (1974) have found M. miehei protease at recommended levels to yield Cheddar and other hard type cheese of satisfactory quality without bitter flavor.

Sternberg (1971) was able to prepare M. miehei protease in crystalline form and he also found cheese made with calf rennet and with M. miehei protease to be indistinguishable. Ottesen and Rickert (1970) showed the great stability of M. miehei protease. Over 90% of the activity was retained after 8 days of incubation at 38°C and between pH 3.0 and 6.0. When incubated at pH 6 for 11 h in 8 M urea, the enzyme lost no activity.

Holmes, et al. (1977) reported that M. miehei and M. pusillus proteases are stable and active during cheese making. At dipping the curd retained 6% of the M. pusillus protease and 4% of the M. miehei protease. Distribution of M. pusillus and M. miehei proteases between curd and whey was not pH dependent, in contrast, distribution of rennet was pH dependent. They also found that 83% of the enzyme activity from Mucor sources remained in the whey and 17% in the curd. This suggests that these enzymes' contributions in cheese curing are minor compared to that of lactic starter

organisms. Duersch (1976) found that M. miehei protease was the most heat stable of all the milk clotting enzymes tested.

Porcine pepsin is an animal protease which has been recommended as a satisfactory substitute for part of the rennet in making a number of varieties of cheese (Emmons, et al., 1970 and Chapman and Burnett, 1968 and Melachouris and Tuckey, 1964). Pepsin is secreted as inactive pepsinogen that has molecular weight of 40,400. Pepsinogen is stable in slightly alkaline or neutral solutions. Below pH 5 pepsin catalyzes conversion of pepsinogen to pepsin (Lee, 1975). Because of instability of porcine pepsin above pH 6.5, slow coagulation of milk could give a weak curd and high fat losses (Emmons, et al., 1970 and Chapman and Burnett, 1968).

Between pH 6.3 and 6.8 the milk clotting activity of porcine pepsin decreases much more rapidly than that of chymosin (Tsugo and Yamauchi, 1959). In fact, at pH 6.8, porcine pepsin may not clot milk (Ernstrom, 1961). Pepsin shows high sensitivity to heat at pH's other than 6.3, so it can be easily destroyed when no longer needed.

Green (1972) reported that Cheddar cheese made from bovine and swine pepsins were only slightly inferior in quality and intensity of Cheddar cheese flavor to rennet cheeses. Ratios of milk clotting activity to general proteolytic activity were high for rennet and bovine pepsin and low for swine pepsins. Bovine mucosa gave low milk clotting activities compared with calf stomach. Bovine pepsin resembles rennet much more closely than does porcine pepsin in many characteristics of importance for cheese making (Fox, 1969b). The

extreme pH dependence of the milk clotting activity of porcine pepsin makes it unsuitable for coagulation of milk of even moderately high pH. This is one reason why mixtures of rennet and porcine pepsin, rather than pepsin alone, are used in cheese making.

The ability of porcine pepsin to degrade β-casein more quickly than either calf rennet, or bovine pepsin may be significant, as many enzymes which are very proteolytic and unsuitable for cheese making, e.g. ficin, papain, Bacillus subtilis protease etc. degrade β-casein very quickly (Fox, 1968) but rennet degrades β-casein slowly and only to a limited extent (Lindqvist and Storgards, 1962; Ledford, O'Sullivan and Nath, 1966; Fox, 1969a). It might be inferred that inability to degrade β-casein extensively is an essential characteristic of rennet and suitable rennet substitutes.

Enzyme Activity and Stability

To obtain accurate measurement of enzymes' activities, it is important to assure their stability. Many factors affect enzyme stability, such as, temperature, pH, ionic activity and specific ions in the substrate (Mickelsen and Ernstrom, 1967).

Prochymosin is stable above pH 7 (Kleiner and Tauber, 1932). At room temperature it is stable from pH 5.3 to 9.0 (Foltmann, 1971). In contrast, the optimum pH for chymosin stability is between 5.3 and 6.3. It is unstable near pH 3.5 and moderately stable at pH 2.0 (Foltmann, 1959). Mickelson and Ernstrom (1967) found that at pH 3.5 chymosin was even less stable in the presence of sodium chloride. Instability near pH 3.5 may be due to self

digestion (Foltmann, 1959), since this is near the optimum for the proteolytic activity of chymosin.

Mickelsen and Ernstrom (1972) reported that blends of chymosin and porcine pepsin are most stable at pH 5.5. Below pH 5 chymosin activity is destroyed by pepsin, and above pH 5.5 pepsin becomes progressively less stable. There is no evidence that chymosin affects pepsin activity. The poor stability of chymosin below pH 5 at high ionic activity is at least partly ionic (Mickelsen and Ernstrom, 1967).

Herriott (1962) found that porcine pepsinogen is stable in neutral and slightly alkaline solutions. It undergoes reversible denaturation above 55°C at pH 7 and at room temperature at pH 5. The reaction is catalyzed by pepsin and accompanied by splitting off about 20% of the molecule.

Pepsin is a single chain peptide (Van Vunakis et al., 1957) with very little helical coiling, as judged by rotatory dispersion measurement (Jirgensons, 1958 and Perlmann, 1959). There are only three disulfide bonds in pepsin, and at least one of those is not essential to enzymatic activity (Bovey and Yanari, 1954 and Herriot, 1962). Hydrogen bonding may not play an important role in stabilizing the molecular because 4M urea has little effect on inactivating the enzyme (Perlmann, 1956 and Steinhardt, 1938). Perlmann (1959) noted that pepsin contains nearly double the usual percentage of amino acids with hydrophobic side chains.

Bovine pepsin was prepared in crystalline form by Northrup (1933). Bovine pepsinogen differs considerably in amino acid

composition from porcine pepsinogen (Chow and Kassell, 1968). Fox (1969a) found that milk clotting activity of bovine pepsin is less pH dependent than that of porcine pepsin, and can coagulate milk up to pH 6.9. The ratio of milk clotting activity to general proteolytic activity is higher of rennet and bovine pepsin than of swine pepsin.

Protease from M. miehei is heat stable and survives 1 h at 60°C. M. miehei protease is not metal dependent and does not have serine or SH groups associated with the active site (Sternberg, 1971). Sternberg (1971) isolated this enzyme and found that its maximum stability is between pH 4 and 6. After 8 days incubation at 38°C between pH 3 and 6 over 90% of activity was retained (Ottesen and Rickert, 1970). The enzyme lost no activity when incubated at pH 6 for 11 h in 8M urea.

Protease from M. pusillus was more sensitive to pH change, between pH 6.4 to 6.8 than was chymosin, but much less sensitive than porcine pepsin (Richardson, et al., 1967). Richardson, et al. (1967) reported that M. pusillus protease is more stable than chymosin between pH 4.75 and 6.25. Mickelsen and Fish (1970) found M. pusillus protease much less proteolytic than E. parasitica protease and more proteolytic than chymosin.

E. parasitica protease shows maximum stability between pH 3.8 and 4.8. Below pH 2.5 activity losses are due to autolysis of the molecule (Whitaker, 1970). Sardinas (1968) found that E. parasitica protease is completely destroyed in 5 min at 60°C and pH 4.5. Changes of pH in milk do not affect milk clotting activity of

E. parasitica protease as much as they do the activity of chymosin (Larson and Whitaker, 1970 and Reps, et al., 1970). The ratio of milk clotting activity to proteolytic activity of this enzyme is lower than that of chymosin (Annibaldi, et al., 1970).

In 1976, Duersch reported that protease from M. miehei is more heat stable between pH 5.2 and 7 held between 68.3 and 73.96°C for .25 to 10 min in Cheddar cheese whey than, in decreasing order of stability, M. pusillus protease, rennet, bovine pepsin, E. parasitica protease and porcine pepsin. As the pH is increased the heat stability of all enzymes except E. parasitica protease decreases. E. parasitica protease stability decreases with decreasing pH. All enzymes except E. parasitica protease are inactivated at pH 7 with minimum heat treatment. M. miehei protease persists after 10 min treatment at pH 5.2 and 73.9°C. High cooking temperature inactivates E. parasitica and therefore doesn't effect cheese during ripening (Whitaker, 1970 and Sardinas, 1968).

Factors Affecting Coagulation and Enzyme Clotting Ability

Many studies have been conducted to find the effect of different factors in milk clotting enzyme stability and clotting ability. Instability of enzyme always results in activity loss, Therefore, the factors which participate in enzyme instability must be controlled as much as possible.

Effects of pH, temperature, calcium, and substrate on coagulation have been studied. Cheryan, et al. (1975) found that decreasing pH from 6.7 to 5.6 causes a 30 fold decrease in clotting

time. Effect of pH on the enzymatic phase of milk coagulation is minor compared to its effect on aggregation. Rate of increase of curd firmness following coagulation rises as pH is decreased from 6.8 to 6.3 (Kowalchyk and Olson, 1977). Acidification with HCl reduces clotting time as a result of changes of Ca^{+2} activity (Ernstrom, 1961). Shalabi and Fox (1982) reported that direct acidification results in reduction of colloidal calcium phosphate concentration and increase of Ca^{+2} activity so clotting time is increased.

Aggregation of renneted micelles is dependent on the solution conditions. A decrease of pH from 6.8 to 6.3 accelerates the reaction by a factor of about 2 (Kannan and Jenness, 1961).

Cheryan, et al. (1975) found that decreasing temperature by 10°C reduces the rate of the enzymatic phase by a factor of 2 and the aggregation phase by a factor of 11 to 12. Most enzymatic reactions have temperature coefficients between 2 and 4 (Berridge, 1942). The Q_{10} for the enzymatic phase of milk coagulation at pH 6.7 between 1 and 30°C is 1.8 to 2 (Nitschmann and Bohren, 1955). Temperature is of considerably more importance, causing more than five fold increase between 31 and 40°C (Kay and Dykes, 1977). The effect of temperature is complex, in that the Arrhenius plot for aggregation of completely renneted micelles is not linear but shows a gradually decreasing temperature coefficient as temperature is increased. Proteolysis of κ-casein is unaffected by presence of other caseins but their presence appears to modify its aggregation behavior. In the absence of Ca^{+2}, para-κ-casein appears to

associate with α_s,-and β-caseins and does not precipitate, as it does when κ-casein is isolated (Lawrence and Creamer, 1969 and Berry and Creamer, 1976).

At temperatures less than 8°C, κ-casein proteolysis takes place in the absence of coagulation. Coagulation occurs on subsequent warming. However, extensive proteolysis with immobilized chymosin, even at low temperature, is not sufficient to prevent aggregation (Dalgleish, 1979). Kowalchyk and Olson (1977) reported that the effect of temperature on aggregation suggests that hydrophobic interactions play an important role in micelle aggregation and formation of gel network.

Addition of $CaCl_2$ to milk reduces the amount of enzyme required for optimum curd formation and decreases clotting time (Ernstrom, 1958). McMahon, et al. (1984) found that addition of $CaCl_2$ up to .05 M causes reduction of clotting time to a minimum. At high $CaCl_2$ (.4M), clotting time is retarded severely, and only weak curd is obtained.

Concentration of substrate plays an important role in coagulation. Concentration of milk by ultrafiltration results in fairly constant coagulation time. However, the amount of casein not incorporated into the curd at clotting time increases (Dalgleish, 1981). Dalgleish (1981) reported that 11% of the casein is not incorporated into the curd in unconcentrated milk. However, 50% is not incorporated in 4X concentrated milk. Dalgleish (1981) also reported that the subsequent behavior of these "free" casein particles may determine final properties of the curd. The

proportion of casein that forms the initial curd decreases as clotting time increases. At clotting time, enzyme concentration required to produce the same proportion of casein incorporated into the coagulum, increases in proportion to concentration factor of the milk (Dalgleish, 1981). Coagulation time is reduced as milk is concentrated.

Coagulation time increases upon dilution of milk and is dependent on dilution factor (Ruegg, et al., 1974). As concentration of milk increases, extent of κ-casein proteolysis at clotting decreases (Garnot and Corre, 1980). The volume of aqueous phase also decreases and shortens the mean free distance between micelles (Garnier, 1973). At low substrate concentration aggregation rate is slow compared to proteolysis. At high concentration overall rate is dominated by rate of enzymatic action (Dalgleish, 1980).

Effect of milk clotting enzymes on coagulation has been investigated by many workers. Milk clotting enzymes from both animal and microbial sources are used in cheese making. The prime action of milk clotting enzymes is proteolysis of specific bond in κ-casein. Beyond that, different enzymes vary in their proteolytic activity on κ-casein and other proteins (Ernstrom and Wang, 1974). Most calf rennet substitutes are more proteolytic relative to their milk clotting activity than is calf rennet. If proteolytic activity is excessive, bitter flavor, cheese yield and fat retention by the curd may be diminished, and during cheese ripening, this can have undesirable effects on cheese body and texture (Green, 1977). Curd firming rates vary from enzyme to enzyme with the more specific

enzymes producing a firm gel more quickly (Kowalchyk and Olson, 1978).

The effect of type of milk clotting enzyme, $CaCl_2$ concentration, and season of year on milk clotting time and curd firmness were studied by Kowalchyk and Olson (1979) using proteases derived from M. miehei, M. pusillus, rennet and mixture of rennet and porcine pepsin. They reported that curd formed by M. miehei and M. pusillus proteases at pH 6.7, 30°C and pH 6.3, 23°C firms less rapidly than curd formed by the other enzymes. Addition of $CaCl_2$ to milk increases the rates of firming of gel formed by all enzymes tested and reduces clotting times. The effect of season on clotting times of milk treated with rennet was not clearly noticeable. Rate of firming of clots formed from milk varied markedly at various seasons. Seasonal variations showed a greater effect on clot-to-cut time than the other factors. Time from clotting to cutting are longer for M. miehei and M. pusillus proteases than for chymosin and calf rennet/porcine pepsin mixture. At pH 6.5, 30°C and pH 6.3, 34°C no significant differences in rates of firming between gel formed by the various enzymes. Gel formed by M. miehei and M. pusillus proteases (at pH 6.4, 30°C and pH 6.5, 34°C) firms less rapidly than gel formed by the other enzymes.

There is about 3 min difference in clot-to-cut time between milk clotted with rennet and with M. miehei and M. pusillus proteases at pH 6.5, 34°C compared to 7.8 and 9.2 min differences between milk treated with M. miehei and M. pusillus proteases and rennet at pH 6.7 and 30°C. Also milk samples treated at pH 6.6 and

31°C with M. pusillus protease firm significantly slower than milk samples treated with other enzymes. These results suggest that it may not be suitable to standardize milk clotting enzymes solely on clotting time since rates of firming of curd from commercial milk supplies vary (Kowalchyk and Olson, 1979).

Hamdy and Edelston (1970) studied the effect of NaCl, $CaCl_2$ and temperature on clotting activities of enzymes from M. miehei, E. parasitica and M. pusillus and from commercial calf rennets. Coagulation time using calf rennet was reduced by addition of NaCl to milk. Adding .2 to .3% NaCl reduced coagulation time to 87% of that when no NaCl was added. Addition of .5 to .7% NaCl to protease from M. miehei .3 to .4% NaCl to protease from E. parasitica, and .4 to .6% NaCl to M. pusillus protease reduced clotting times to 68.8, 86 and 64% respectively. Addition of more than .6% NaCl to calf rennet and more than 1.5% to M. miehei or E. parasitica protease increases clotting time. Addition of .05, .075, .04, and .05% $CaCl_2$ to M. miehei, E. parasitica, M. pusillus protease and calf rennet reduced the clotting time by 50%. Increasing incubation temperature from 37°C to 42°C reduced by one-half the clotting time for M. miehei protease. Microbial rennets are more sensitive to change in temperature from 37°C to 42°C than from 35°C to 37°C. Optimum activity for all four enzymes is 42°C.

Methods for Measuring Chymosin Activity

Several methods have been used to measure chymosin activity. The rate at which the product of enzyme catalyzed reaction appears

or the rate at which substrate disappears is used very commonly in measuring enzyme activity. Milk clotting is a complex process involving two reactions, the primary (enzymatic) and the secondary (non-enzymatic).

It is difficult to create a standard milk substrate for the clotting test because of natural variations in milk composition. The ability of a protease enzyme to catalyze hydrolysis of a specific Phe-Met peptide bond in $_{\kappa}$-casein is used as criteria in measuring the enzyme activity (Delfourd, et al., 1965; and Jolles, et al., 1968) under a set of specified conditions. There are problems in accurately observing the end point of coagulation and standardizing the conditions of the clotting test.

Berridge (1952b) suggested reconstituted non-fat dry milk (12%) as a substrate for measuring milk clotting activity. This substrate is prepared by dissolving 12 g of non-fat dry milk in 100 ml of .01 M $CaCl_2$. Test tubes with 10 ml of substrate are placed in a 30°C water bath for 30 min; then 1 ml of diluted enzyme extract is added to each tube. The clotting time is determined by measuring time from addition of the enzyme until tiny flakes become visible on the inner surface of the test tubes. Berridge later (1952a) improved the technique of detecting the end point of the enzyme reaction. A stirring rod was dipped into the substrate and touched to the side of the test tube. This allows milk adhering to the rod to flow down the side of the test tube forming a flowing film.

Sommer and Matsen (1935) first described an apparatus for measuring clotting time which has been used for many years. The

apparatus consists of a rectangular metal box filled with water at constant temperature (30°C). A number of tilted metal rods rotate and are used to restrain sample bottles on. Wide mouth bottles containing 50 ml of substrate each are rested on the rods, partially submerged into the water. The substrate is inoculated with one milliliter of diluted (1:50) enzyme. Clotting times are measured by timer attached to the apparatus. Times are measured from addition of enzyme until appearance of flakes on the inner surfaces of the rotating bottles.

The combination of Sommer and Matsen apparatus and Berridge substrate in measuring chymosin activity was applied by Ernstrom (1958). He proposed that the substrate be stored at 2°C for 20h before use because it continues to show increase in clotting time for 20h after its preparation when kept at 2°C. The activity of unknown activity chymosin extract is determined by measuring the required amount of time for one milliliter of an appropriate dilution of the unknown extracted to clot 25 ml of Berridge substrate. This is compared to the time required for 1 ml of known dilution of standard activity rennet extract to clot the same amount of an identical substrate. The activity of undiluted standard chymosin is arbitrarily assigned the value of 100 chymosin units (CU) per milliliter. The following equation is used to calculate the activity of an unknown solution.

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

where

CU/ml = chymosin units/ml of unknown

100 = chymosin units in one ml of undiluted standard chymosin

Ts = coagulation time with standard chymosin

Tu = coagulation time with enzyme of unknown activity

Cs = concentration of standard enzyme

Cu = concentration on unknown enzyme

DeMann and Batra (1964) used an automatic blood clotting timer in measuring chymosin clotting activity in milk. This method requires less substrate and has an automatic end-point detector, and it is faster than the Sommer and Matsen method. The ratio of enzyme solution to substrate is higher than is used with most other clotting tests. Therefore, care must be exercised to prevent differences in pH or salt concentration in the enzyme solution from affecting the clotting time.

The clotting point can also be determined by measuring the increase in velocity of ultrasonic energy passing through a sample (Everson and Winder, 1968).

Douillard and Ribadeau-Dumas (1970) used the clotting time of κ-casein solution for measuring the activity of chymosin, porcine pepsin, and bovine pepsin. They reported that precaution must be taken to control ionic activity, pH, and κ-casein concentration in order to obtain an accuracy of 1% within a single laboratory.

Change in milk viscosity was used as an indicator of clotting time by Scott-Blair and Oosthuizen (1961). A difficulty which often affects this method is that plots of changes in specific

viscosity versus time of chymosin action on caseinate solution always give the same slope, but the slope varies with different lots of rennet extract. This has been attributed to differences in protease content between the extracts.

Gorini and Lanzavecchia (1954) devised a sensitive substrate which was modified by Wang (1969) and used by Reyes (1971) to measure residual chymosin in curd and whey. Reyes' (1971) procedure is nearly 12 times more sensitive than the Berridge method and facilitates measurement of chymosin activities as low as .01 CU/ml.

An assay method involving linear diffusion of milk clotting enzymes through a casein-agar diffusion substrate was developed by Holmes and Ernstrom (1973). Holmes (1974) was able to measure rennet at concentration as low as 1×10^{-4} CU/ml. Casein-agar substrate was put into 3 mm x 110 mm sedimentation tubes. This substrate consists of .5% casein, .01% $CaCl_2$, .7% ion agar and 3.6% sodium acetate. To the surface of the substrate in each test tube, 5 µl samples of enzyme solution are deposited and allowed to diffuse through the casein agar. As the enzyme diffuses through the substrate, a white precipitation band is formed as a result of action of enzyme on casein. Diffusion distance is proportional to enzyme concentration. Density of the precipitate at the leading edge of the band is a function of casein concentration and is independent of enzyme concentration. The disadvantage of this method is incubation for 48h before measuring activity. In addition, use of a highly purified agar is required due to sensitivity of whole casein to calcium. Clouding is frequently

produced in the diffusion tubes when using whole casein and $CaCl_2$ in the substrate. This makes detection of diffusion distance difficult. This was eliminated by replacing whole casein with κ-casein, eliminating the $CaCl_2$ and raising the pH to 5.9 (Duersch, 1976). The advantages of this method over other methods of enzyme analysis are greater sensitivity, easy measurement of diffusion distance with a densitometer and no effect of whey solids or salt on diffusion.

The Formagraph instrument has been evaluated and used in measuring milk clotting ability (McMahon and Brown, 1982, 1983). It consists of ten adjacent sample wells in a rectangular block. This block moves slowly from side to side. A pendulum with a wire loop on the end is immersed into the sample wells. This remains vertical as long as the milk in the well is liquid. When the milk starts to clot, the pendulum tilts as the heating block moves. A straight line appears on the graph paper as long as the sample are not coagulated. Once the milk starts to clot, the pendulum begins to tilt as the heating block moves. McMahon and Brown (1983) suggested that this method can measure milk clotting enzyme activity within the range of the gel diffusion test of Holmes et al. (1977).

McMahon, et al. (1984) reported that the clotting activity and coagulation properties could easily be followed using a Beckman DU-8B UV/vis spectrophotometer with a temperature controlled cuvette holder and light scattering accessory. They monitored changes in apparent absorbance at 600 nm of milk as a result of clotting enzyme action.

Methods for Protein Determination

Protein content assay of any substance usually depends on determining a specific element or group in the protein, and calculating the protein content by multiplying by an experimentally determined factor. Methods based on analysis for constituents of proteins include those for determining carbon or nitrogen, certain amino acids, or the peptide linkage. Iron in hemoglobin, iodine in thyroglobulin and nitrogen in milk and some other foods can be used as basis for protein assays. In all these methods, it is assumed that the constituent determined is present entirely in the protein fraction. Thus, any nonprotein carbon-containing matter must be removed if the protein content is to be determined from the carbon content; and if the Kjeldahl is used, protein-nitrogen only should be measured. The common practice of estimating protein content of food from total nitrogen is not always correct. Presence of nonprotein nitrogen compounds is generally small compared to the protein content of most foods.

Carbon analysis has several advantages over other methods for determining protein content in foods. Digestion can be done easier than for nitrogen determination, and high percentage of carbon minimizes experimental error and provides a relatively constant conversion factor. The disadvantage is difficulty of a complete quantitive separation of protein from nonprotein carbon-containing components.

The most commonly used procedure for protein assay is nitrogen determination. It is generally assumed that a mixture of pure

proteins contains 16% nitrogen. Reporting protein content confuses people because of different conversion factors which are used by different laboratories. To eliminate this confusion, nitrogen percent rather than protein content has been used. The general factor of 6.25 (100/16) is used for most foods. For milk and meat the factors are 6.38 and 5.7 respectively (Jones, 1931). Protein isolation and characterization, including area composition, provides the basis for a continuous reexmaination of the conversion factors (Tkachuk, 1966).

Kjeldahl (1883) invented a method for determining organic nitrogen in his study on protein changes in grain used in the brewing industry. This method was modified by Bradstreet (1940, 1965). Digestion with sulfuric acid continues until carbon and hydrogen are oxidized and protein nitrogen is reduced and converted into ammonium sulfate. After that, sodium hydroxide is added and the sample is distilled to release ammonia into a known volume of a standard acid solution. Unreacted acid is determined and the results are transformed, by calculation, into a percentage of protein in the original sample. Potassium sulfate is used as catalyst to accelerate the digestion with sulfuric acid, raise the boiling point of the digestion mixture and shorten the reaction. Excessive ratios of potassium or sodium sulfate to acid may result in heat decomposition and loss of ammonia. Usually, digestion temperatures of 37° to 41°C are best. Histidine and tryptophan rich protein require long or severe digestion conditions.

Mercury, copper and selenium have been used very widely as catalysts in Kjeldahl digestion. Mercury is superior to copper, but additional steps are required to precipitate the mercury. Mercury-ammonia complex which forms during digestion can be decomposed by adding sodium thiosulfate to the digest. Selenium has more rapid effect than mercury and requires no further treatment before distillation. However, excessive amount of selenium and uncontrolled digestion temperature would cause nitrogen loss.

Several methods are available to determine the ammonium sulfate in the digest. The digest may be alkalized and the liberated ammonia absorbed in acid measured titrimetrically or calorimetrically (Van Slyke and Hiller, 1933). Colorimetric method consists of reacting a solution containing ammonium ions with alkaline phenol and hypochlorate. On heating the solution an intense blue color is produced, which is closely related to that of indophenol (Mann, 1963; Varley, 1966). With the development of a continuous digestion module (Ferreri, 1960), it is possible to determine nitrogen in biological fluids or suspensions within several min. The digestion followed by a colorimetric determination in a neutralized digest to which alkaline phenol and sodium hypochloride are added. An automated Kjeldahl analyzer for determination of nitrogen in biological material was described by Siriwardene, et al. (1966). This methods is capable of handling 20 samples an hour, reduces the labor involved to a minimum, and still maintains a high degree of accuracy and reproducability.

In the classical Dumas procedure (1831), nitrogen was freed by pyrolysis, and free elemental nitrogen was determined volumetrically. Precise and accurate analysis of nitrogen in organic materials has been ensured by major improvements in both the pyrolysis and nitrogen determination (Sternglanz and Kollig, 1962). Improved catalysis and rapid gas chromatographic methods for nitrogen determination make it possible to assay on a microscale in 2 min. In 1965, a fast neutron activation analysis was developed by Wood for analyzing the nitrogen content of foods. In spite of the large initial cost of installation, it shows promise for precise and rapid (about 5 min) assay of protein in various foods.

A simple, rapid and inexpensive procedure, the biuret method, was proposed by Riegler (1914). Compounds containing two or more peptide bounds take on a characteristic purple color when treated with dilute copper sulfate in alkaline solution. The name of this method comes from the compound biuret, which typically gives a positive reaction. The color is apparently caused by the coordination complex of the copper atom and four nitrogen atoms, two each from two peptide chains. This method is fairly reproducible for any protein, and it gives an accurate estimate of protein, but requires relatively large amounts of protein (1-20 mg) for color formation. In contrast, Kjeldahl procedure measures total nitrogen and does not distinguish between protein and non-protein nitrogen (Miller and Johnson, 1954).

Phenol-reagent method is used widely for determination of protein in solution and dried material. This method is based on

interaction of proteins with phenol reagent and copper under alkaline conditions (Wu, 1922). Important modifications of this procedure was done by Folin and Ciocalteau (1927) and Lowry, et al. (1951). The color formed by Folin-Ciocalteau reagent is caused by reaction of protein with alkaline copper in the reagent and reduction of phosphomolybdate-phosphotungstate salts in the reagent by the tyrosine and tryptophan of proteins. This method is more sensitive than ultraviolet absorbance and the biuret method. The method is relatively specific, since few substances encountered in biological materials cause serious interfere (Solecka, et al., 1968). This method is more time consuming than direct absorbance measurement at 280 nm, is destructive, and requires multiple operations on each sample and incubation between additions of reagents.

Warburg and Christian (1941) used the direct spectrometric method (UV 280 nm) as a rapid and fairly sensitive protein determination. Most protein shows a maximum ultraviolet absorbance at 280 nm due to presence of tyrosine, tryptophan and phenylalanine. The combined level of these amino acids in proteins differs within a reasonably narrow range. Concentration of protein (in pure solution) is generally proportional to absorbance at 280 nm. Such assays are advantageous because they are rapid and they allow full recovery of the assayed protein.

Fraenkel-Conrat and Cooper (1944) reported for the first time that proteins bind quantitatively, under specified conditions with certain organic dyes. This method is called dye-binding. Dye

binding can be used to determine total acidic and basic groups of proteins. Specific group reagents for proteins were reviewed by Olcott and Fraenkel-Conrat (1947) and by Rosenberg and Klotz (1960). Disulfonic anionic dye, Orange G is bound at pH 2.2 (Udy, 1954, 1956). This dye binds specifically under acidic conditions to free amino groups, lysine, the imidazole group of histidine, and the guanidino group of arginine. Protein estimation by dye binding has been improved by using acid orange 12 dye, that is structurally identical to orange G with the exception that acid orange 12 has only one sulfonic acid group. Quantitative reaction occurs between the dye and protein to form an insoluble complex. Binding capacity can be calculated by measuring the concentration of unbound dye colorimetrically. The dye binding procedure is rapid and eliminates the problems of skillful manipulation and corrosive reagents of the Kjeldahl procedure.

Additional dyes (cochineal red A, buffalo black, and amido black 10B) have been recommended, mainly for meat protein determination and milk products. Amido black 10B gives greater change in optical density (per unit of milk protein) than orange G (Tarassuk, et al., 1967). The dye binding capacity of milk protein is not affected by homogenizing, condensing, or heating to 90° for 15 min. Extensive proteolysis increases dye binding, whereas heating to browning reduces it. The dye binding test is considered suitable for normal milk samples, but not for atypical milk such as colostrum, mastitis, and very late lactating milks (Tarassuk, et al., 1967).

It was reported in 1964 that formal titration can be used in determining the protein content of natural and processed milk including ice cream (Drux and Bauer, 1964; Hill and Stone, 1964).

EXPERIMENTAL PROCEDURES

Enzymes

Five different commercial enzymes were used: calf rennet, chymosin/pepsin mixture, bovine rennet, M. miehei protease, and modified M. miehei protease. These enzymes were obtained from Marschall Division, Miles Laboratories, Inc. A purified calf rennet extract with 100 chymosin units (CU) of activity per milliliter was obtained from the New Zealand Cooperative Rennet Co., Ltd., Eltham, New Zealand. All dilutions were with distilled water and diluted enzymes were maintained at below 2°C throughout the experiment.

Separation

The five enzymes were fractionated using Sephadex G-100. Samples of one milliliter of each enzyme were applied to a Sephadex G-100 column (50 cm x 1.5 cm), which had been equilibrated with pH 5.7 phosphate buffer. Gel columns were prepared by dissolving 4 g G-100 in 500 ml water and holding it in a boiling water bath for 5 h. Fractions of 200 drops each were collected in 30-40 tubes from each enzyme. Fractionation was done in a cold room (5°C). Each fraction was tested for milk clotting ability and for coagulation properties. In addition, each fraction was tested for protein lost in whey.

Clotting Test

Substrate. Low heat non-fat dry milk was used as substrate in determining milk clotting ability of enzyme samples. This substrate

was prepared by reconstituting 12 g of non-fat dry milk powder in 100 ml of .01 M $CaCl_2$ (Berridge, 1952b). The substrate was kept for 20 h at 5°C before use.

Measurement of Clotting Activities. The clotting ability of commercial enzymes and of those fractions eluted from the column were measured using a Formagraph instrument as described by McMahon and Brown (1982). Times required for coagulation of substrate with commercial and fractionated enzymes were compared to times required for known dilutions of a standard rennet extract to coagulate substrate under the same conditions. All tests were run in duplicate and standard rennet extract dilutions were tested simultaneously with the enzyme fractions.

Protein Determination in Whey

The concentrations of the five coagulants were adjusted so they were all at the same activity. A sample of 500 l of each coagulant was added to 100 ml of pasteurized whole milk. The temperature of the milk was brought to 35°C before adding the enzyme. Milk and coagulant were mixed by stirring and held at 35°C for 30 min. The curd was then cut with a spatula and held in the whey at 37°C for an additional 2 h. The whey was then drained through three layers of nylon filter and whey nitrogen was measured by Kjeldahl. Commercial and purified enzymes were adjusted to the same activities as pairs. Protein lost in whey from each fractionated and commercial enzyme pair was compared.

Protein lost in whey was determined by Kjeldahl procedure (Association of Official Analytical Chemists, 1980). Five grams of sample was digested with 2 g of Na_2SO_4 plus 2 ml mercuric sulfate (10 g in 12% H_2SO_4). Digestion continued for 2 h. Distillation was for 2 min then each sample was titrated with HCl (.0903 N). A mixture of methyl red and bromocresol green indicator was added to boric acid which was used to receive the ammonia distillate. Titration ended when a pinkish-grey endpoint was reached. Percent nitrogen was calculated from molarity of acid, sample weight, volume of acid used in titration after subtracting volume of acid used to titrate the blank sample, and the molecular weight of nitrogen.

Estimates of whey protein were made by multiplying whey nitrogen by 6.38. This whole procedure was repeated 23 times with each of the five commercial coagulants. Each pair of commercial and fractionated coagulants was compared with each other after they had been tested 18 times.

Testing Coagulation Properties

Coagulation of each commercial and improved enzyme was followed using a Beckman DU-8B UV/Vis Spectrophotometer. Three milliliters of Berridge substrate was placed in a 1 cm path length cuvette and heated to and maintained at 35°C. Spectrophotometer absorbance was set to zero with slit width at 2 nm. The reaction mixture was stirred rapidly (<10Sec), then changes of apparent absorbance at 600 nm were recorded every 4 sec and transmitted to a Tektronix 4052 micro-computer for analysis.

RESULTS

Protein recovery and coagulation properties of the five commercial and purified enzyme preparations were determined. Percent protein lost in whey was used as an indication of protein recovery in curd obtained by the five pairs of commercial and improved enzymes. An example of the coagulants fractionation on a Sephadex G-100 column is shown in Figure 1 (Shaker, 1983). The shaded peak represents the portion of the elution curve which clots milk.

Figure 2 illustrates a comparison of protein lost to whey from the five commercial clotting preparations. Analysis of variance showed that there were significant differences among the five coagulants. Duncan's Multiple range test showed that calf rennet/porcine pepsin mixture and M. miehei protease were not significantly different from each other and that bovine rennet, calf rennet and modified M. miehei protease were not different from each other, but that the two groups were significantly different from each other.

Each of the five coagulants and the improved preparations made by selecting the clotting fractions and discarding all others fractions were tested for clotting activity with the Formagraph. Concentrations were adjusted to match clotting activities of original enzyme preparations with the purified samples made from them. Each pair was then used to clot milk to obtain whey. The whey from each pair was then tested 18 times and analysis of

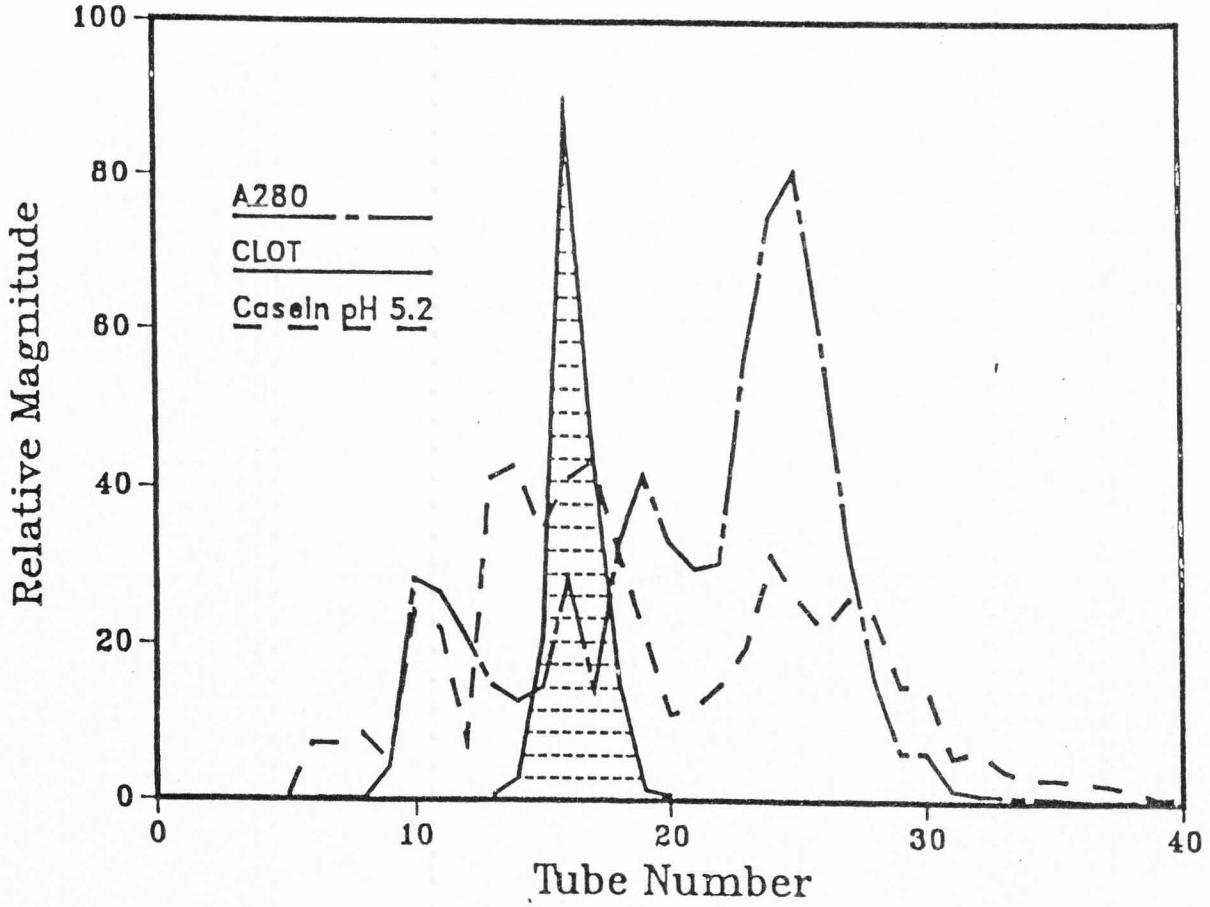

Figure 1. Fractionation of bovine rennet on Sephadex G-100. The curves represent A_{280}, milk clotting activity, proteolytic activity toward hemoglobin at pH 5.2 (Shaker, 1983).

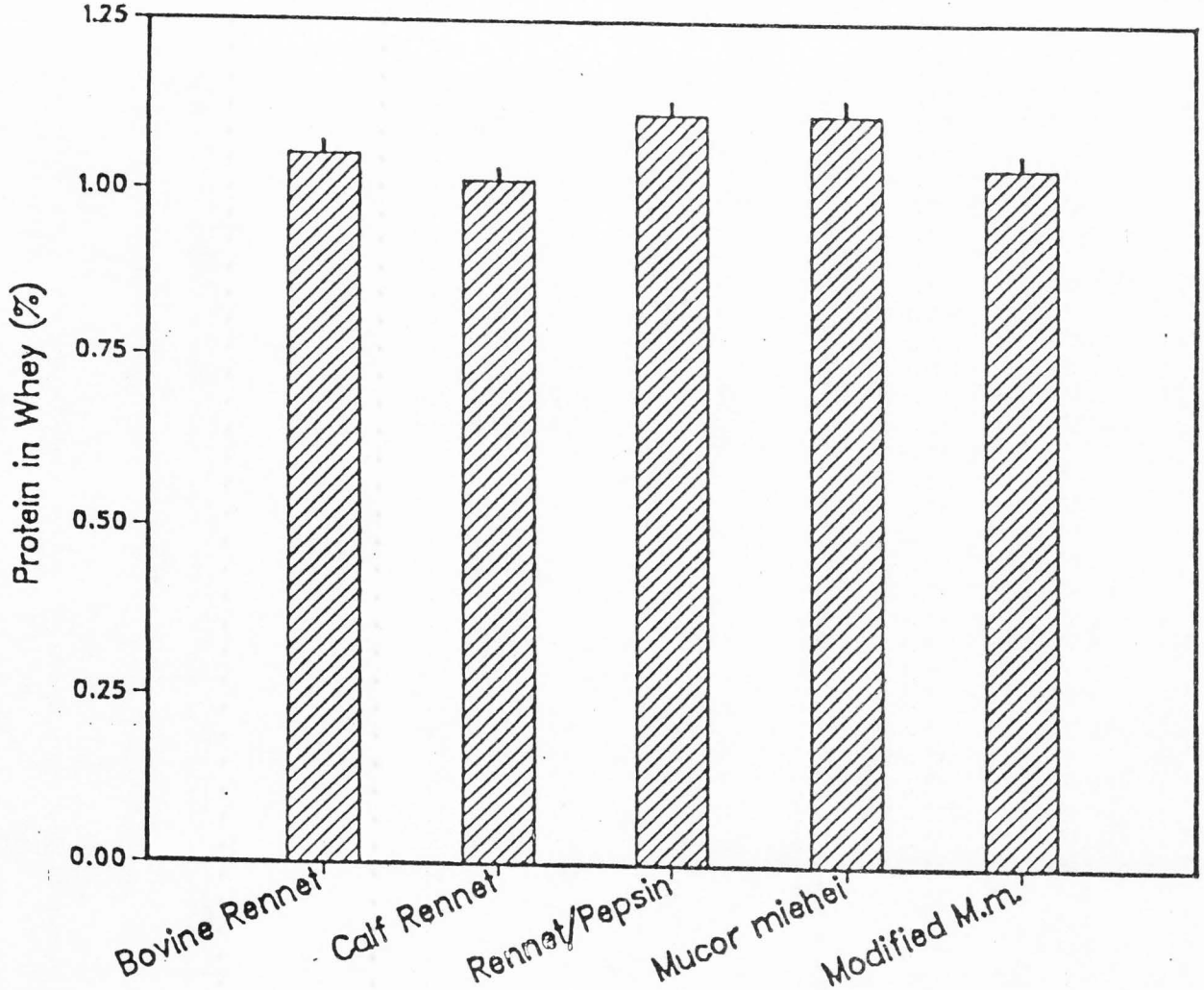

Figure 2. Percent protein lost in whey from five commercial milk clotting enzyme preparations. Lines on the top of the bars represent standard errors of means.

variance were used to determine the significance of differences between original and purified preparations in protein lost to whey.

Percent protein lost to whey from original and improved calf rennet preparation are shown in Figure 3. Original calf rennet preparation resulted in more protein lost to whey than the improved enzyme preparation. However, there was not a significant decrease in protein loss by purification of this enzyme. Commercial preparations has a mean of 1.03 and standard error of mean of .014. Mean and standard error of mean of the improved preparation are .98 and .015 respectively.

As shown in Figure 4, bovine rennet was improved significantly by purification. Means of protein lost to whey from original and improved enzyme were 1.034 ± .014 and .985 ± .015 respectively.

Figure 5 illustrates significant differences in percent protein loss to whey when commercial and purified calf rennet/porcine pepsin mixture were used to coagulate the substrate. The commercial preparation has a mean of 1.05 and standard error of mean of .02. The improved enzyme resulted in less protein lost to whey. The mean for this enzyme is .98 and the standard error of mean is .017. This enzyme was improved significantly by gel filtration.

Commercial and purified M. miehei protease preparations did not show significant differences in percent protein lost to whey. Figure 6 represents the means and standard error of the mean of percent protein lost to whey from the original and improved enzyme. The mean for the commercial and purified enzyme are 1.05 and 1.033 respectively. Standard errors of means were .017 and .0177 respectively.

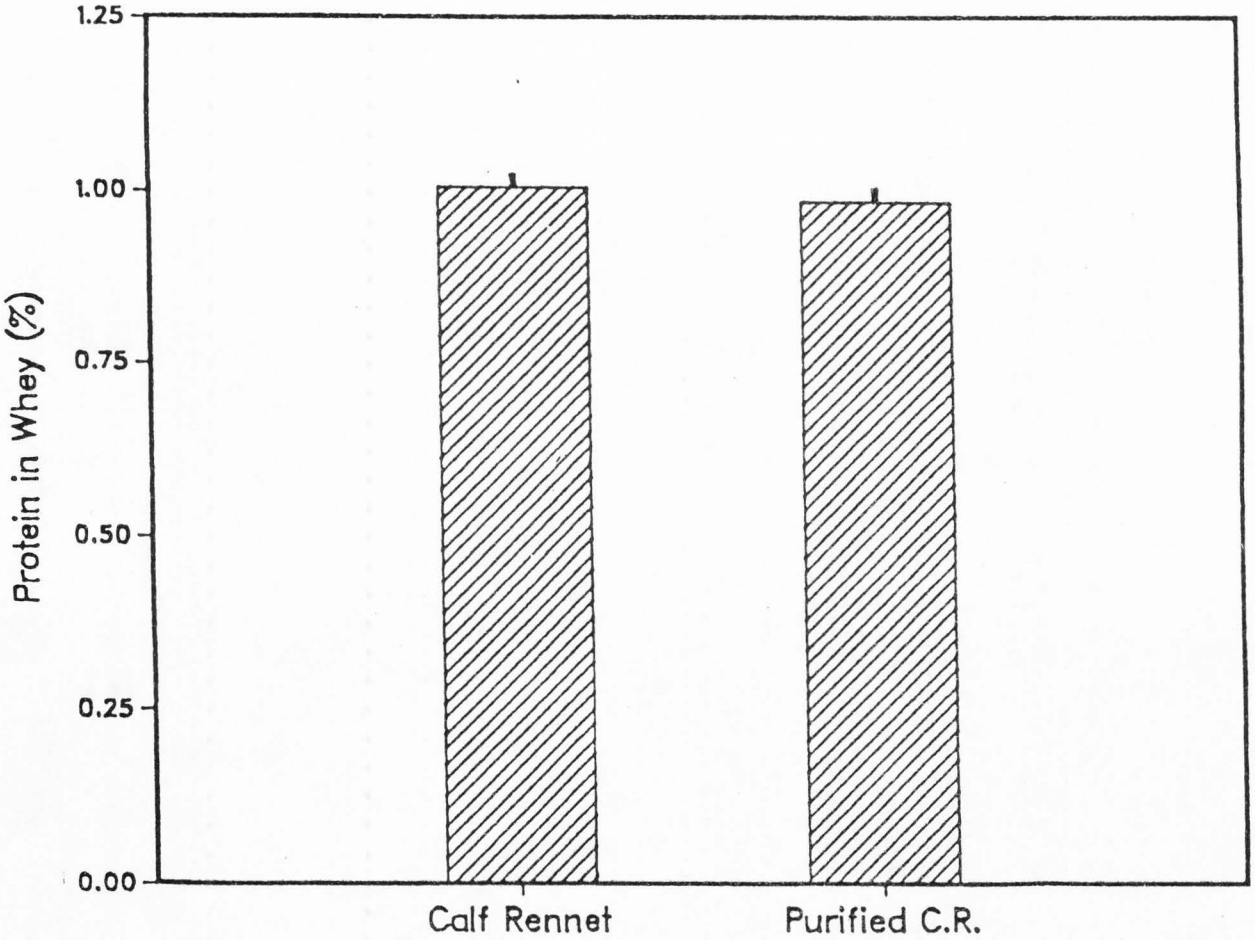

Figure 3. Percent protein lost in whey from commercial and purified calf rennet. Lines on the top of the bars represent standard errors of means.

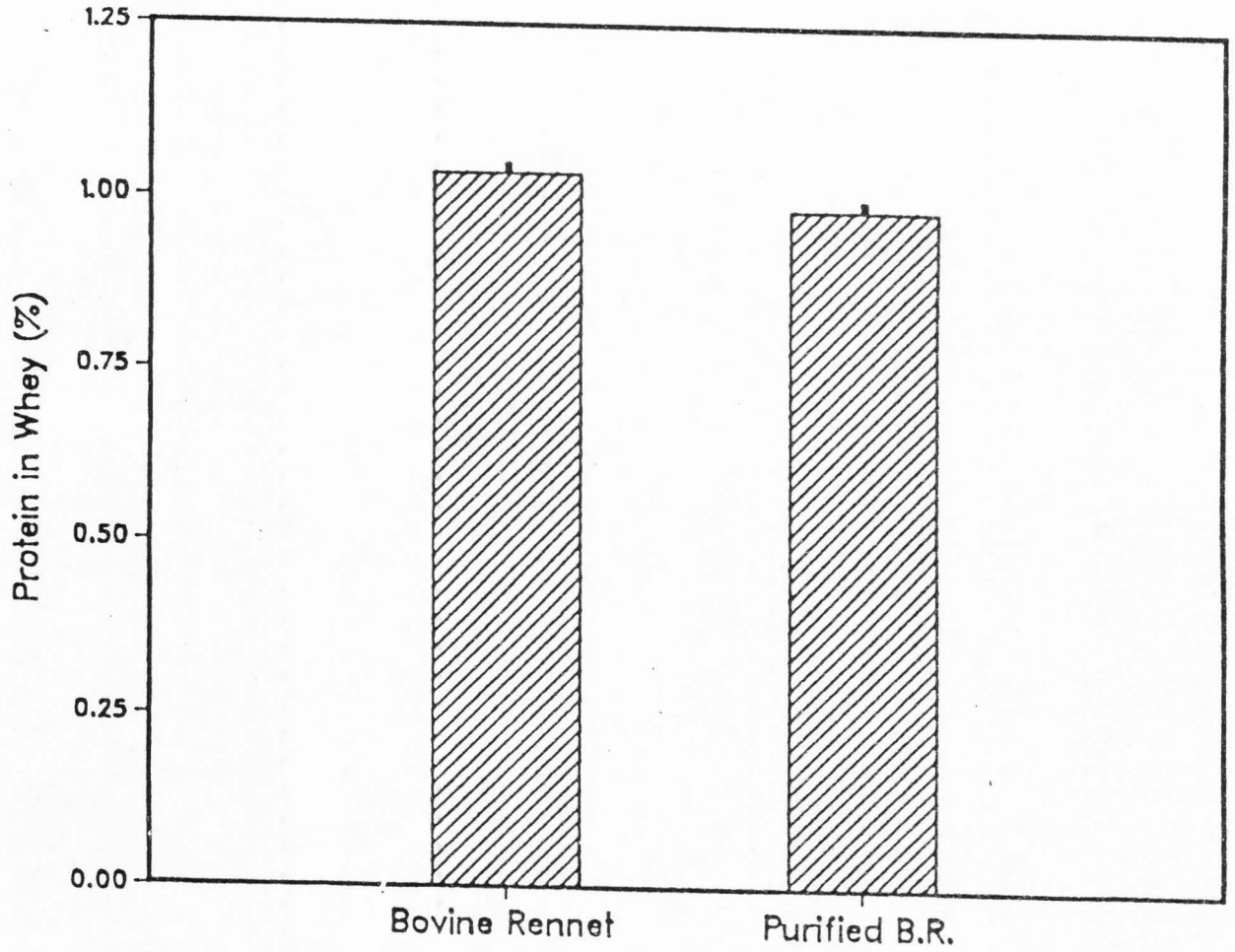

Figure 4. Percent protein lost to whey from commercial and purified bovine rennet. Lines on the top of bars represent standard error of means.

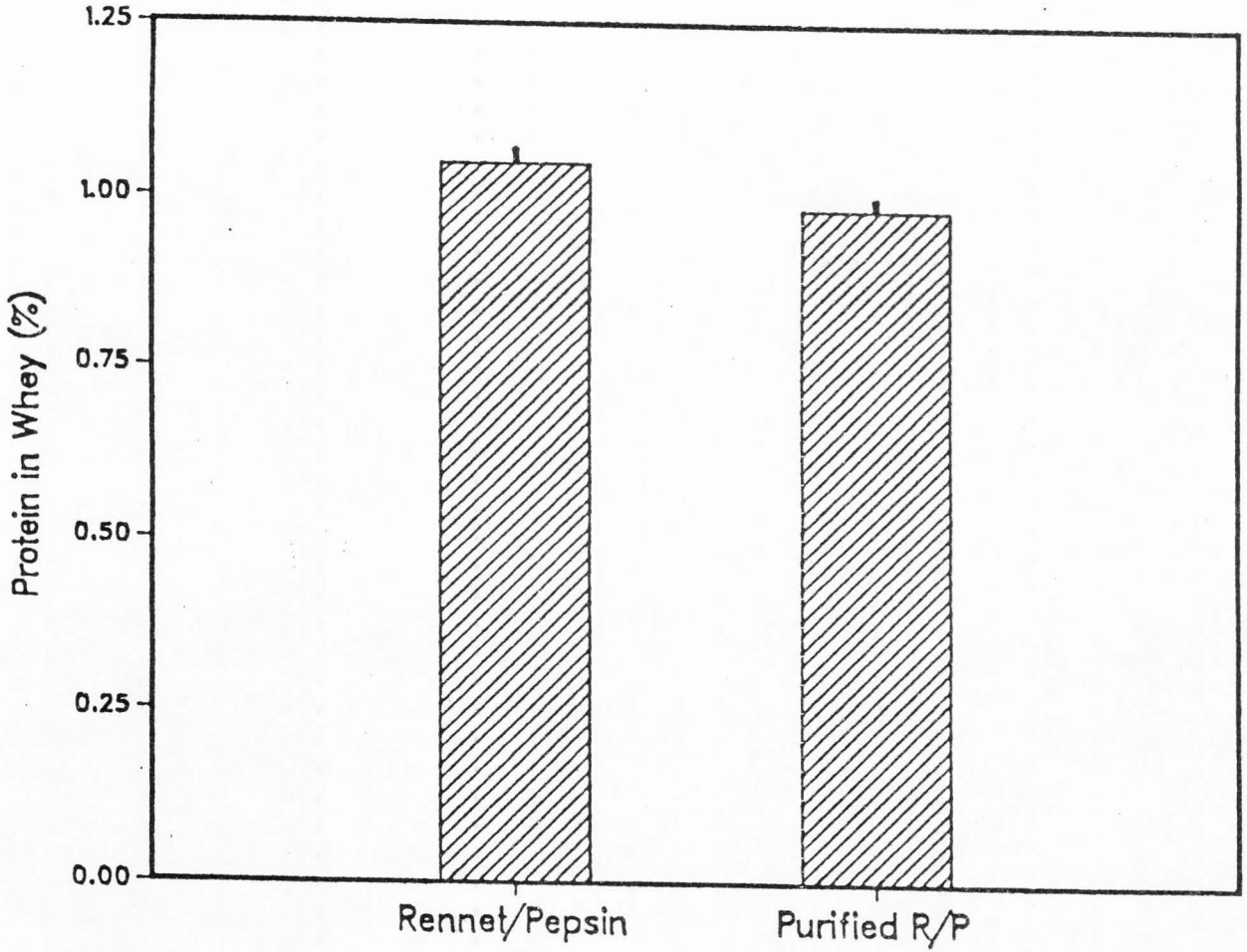

Figure 5. Percent protein lost to whey from commercial and purified calf rennet/porcine pepsin mixture. Lines on the top of the bars represent standard errors of means.

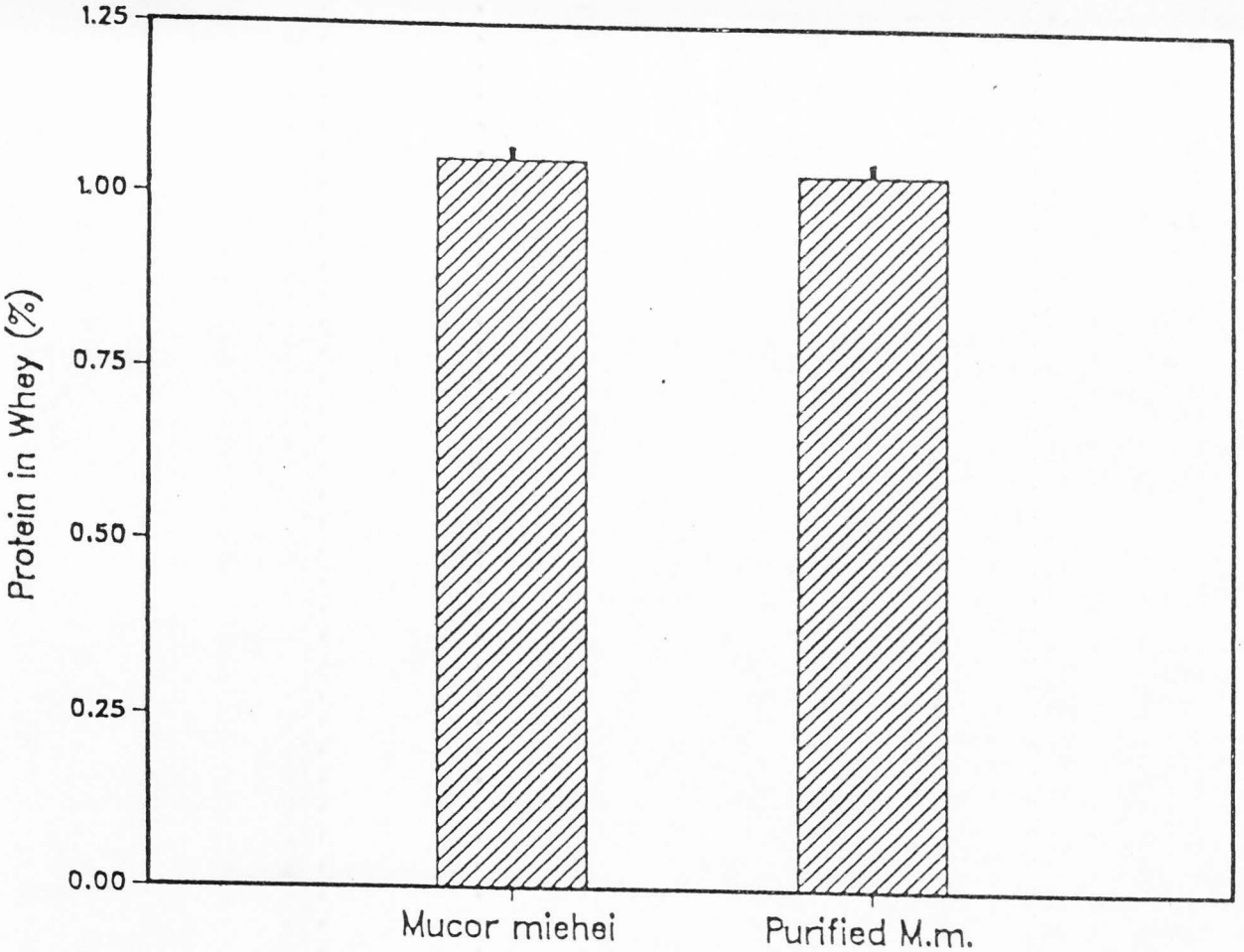

Figure 6. Percent protein lost to whey from commercial and purified M. miehei protease. Lines on the top of the bars represent standard error of means.

Modified M. miehei protease was improved by gel filtration but not significantly. As shown in Figure 7, the commercial enzyme preparation yielded a higher percentage of protein lost to whey than the one from the improved preparation. The commercial preparation had a mean of 1.05 with standard error of mean .02. The improved enzyme had a mean of 1.003 with standard error of .016.

The coagulation curves of the commercial and improved enzyme preparations were followed through their various phases by monitoring apparent absorbance of milk at 600 nm. Figure 8 shows the coagulation of the five commercial enzymes. The concentrations of these enzymes were adjusted to equal milk clotting activities. Figure 9 is an expansion of the first part of Figure 8.

Coagulation of Berridge substrate with each pair of commercial and improved enzymes was followed and is illustrated in Figures 10 through 14. Figure 10 represents the coagulation properties of purified and commercial calf rennet. Coagulation of substrate with bovine rennet, both commercial and purified, is shown in Figure 11. The coagulation properties of commercial and purified calf rennet/porcine pepsin mixture, M. miehei and modified M. miehei protease are shown in Figures 12, 13 and 14 respectively.

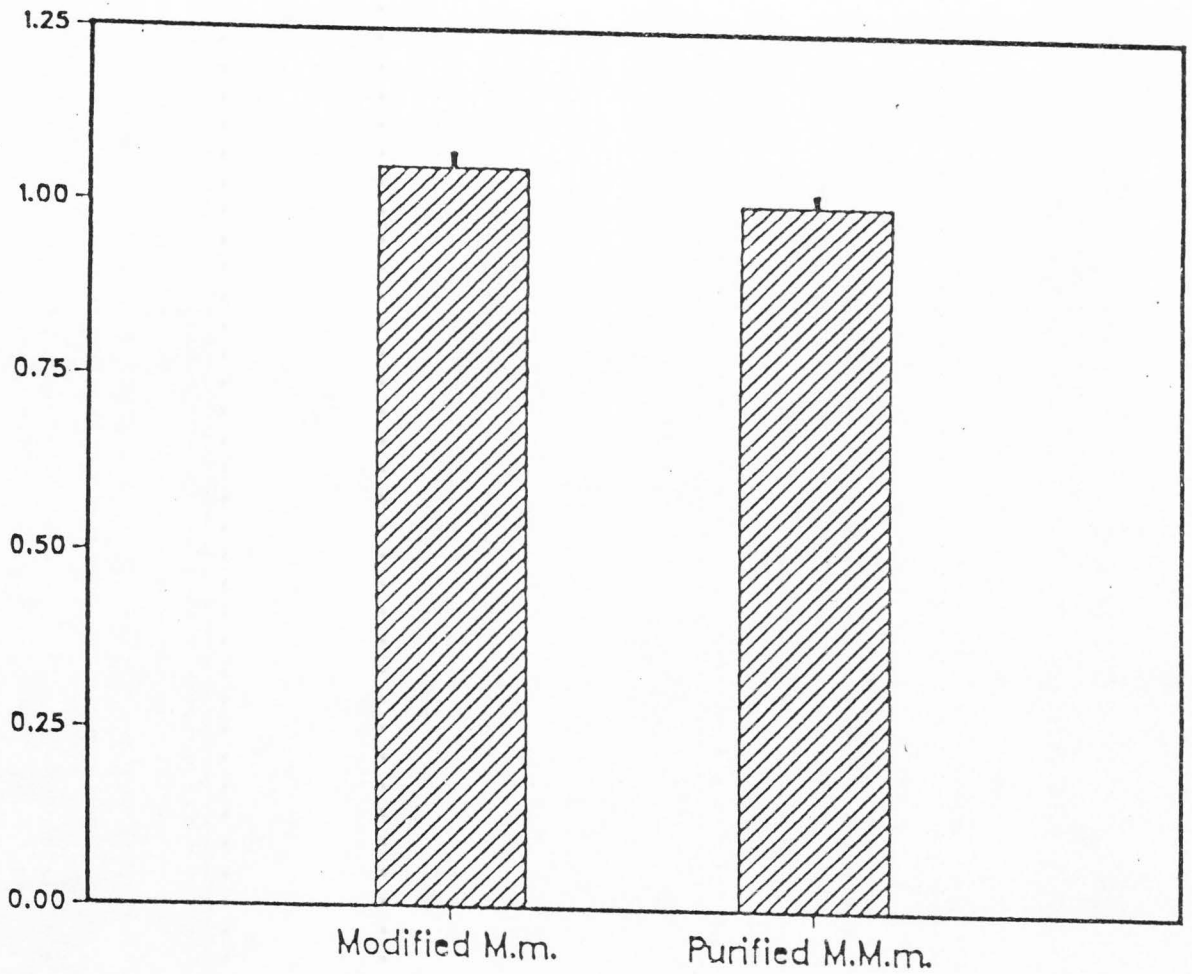

Figure 7. Percent protein lost to whey from commercial and purified modified M. miehei protease. Lines on the top of the bars represent standard error of means.

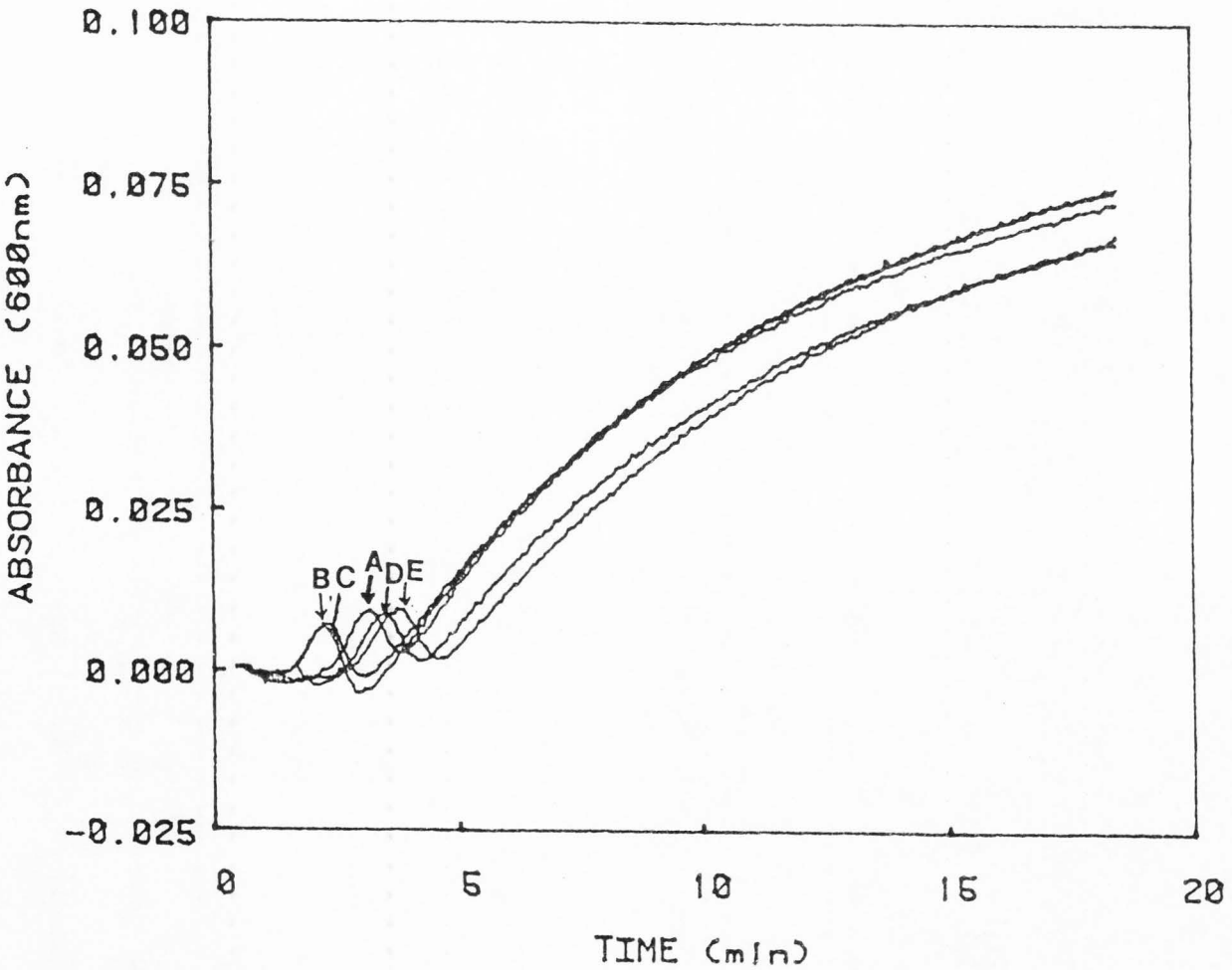

Figure 8. Coagulation curves of five different commercial milk clotting preparations. B, bovine rennet, C, calf rennet/porcine pepsin mixture, A, calf rennet, D, M. miehei protease, E, modified M. miehei protease. Dependent axis represents relative apparent absorbance.

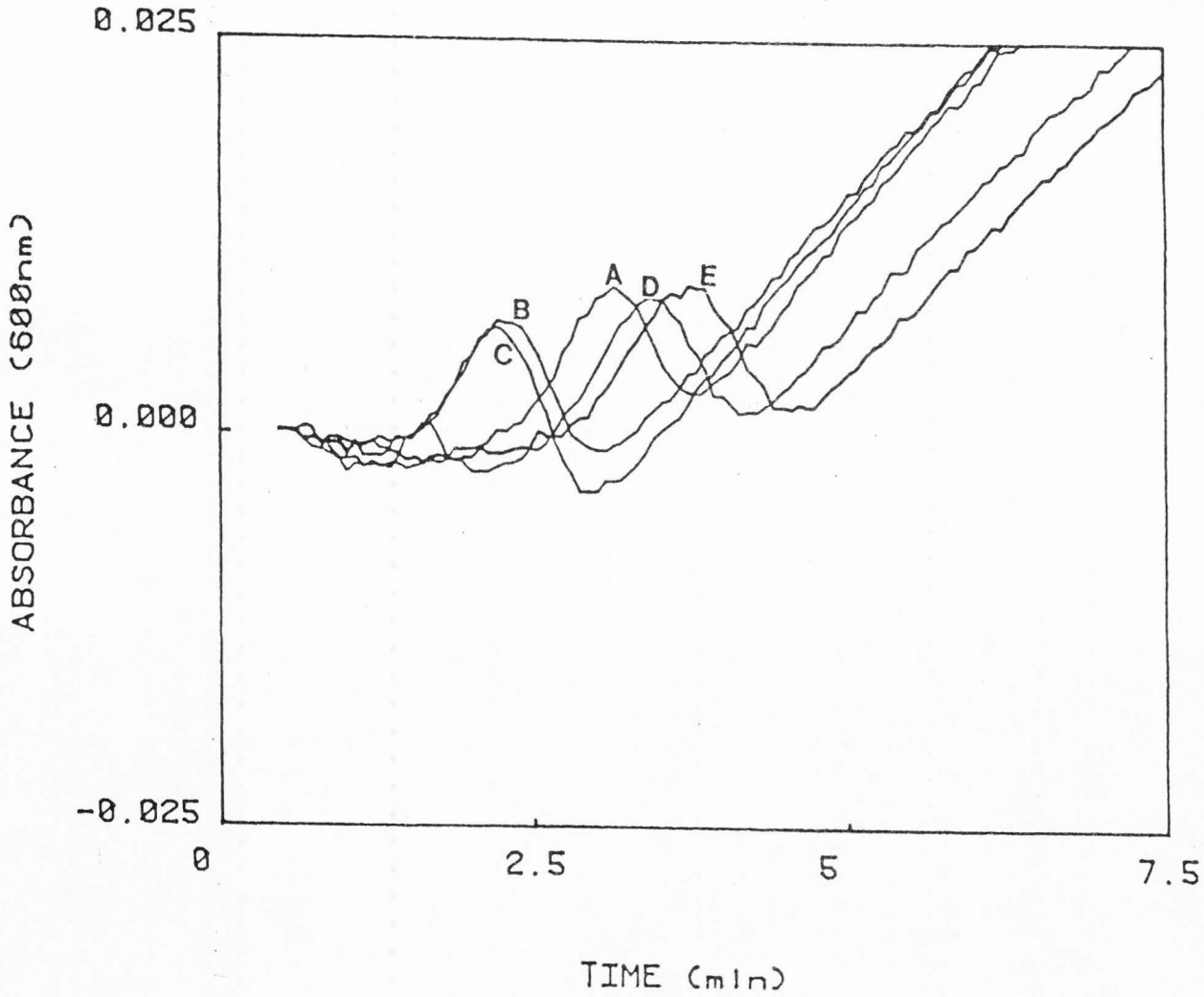

Figure 9. Coagulation curves of five different commercial milk clotting preparations. A, bovine rennet, B, calf rennet/porcine pepsin mixture, C, calf rennet, D, M. miehei protease, E, modified M. miehei protease. Dependent axis represents relative apparent absorbance.

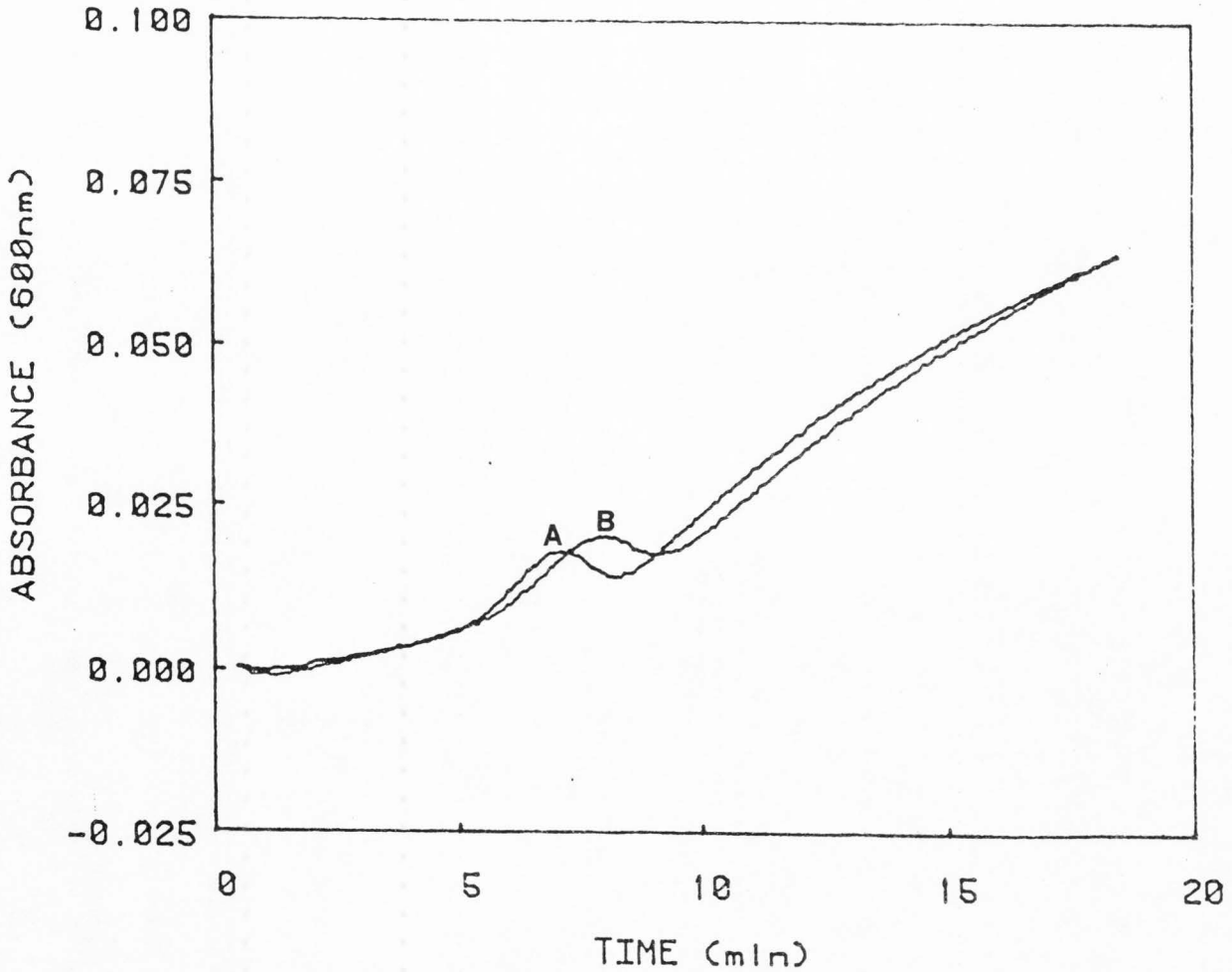

Figure 10. Coagulation curves of the purified and commercial calf rennet. A, commercial calf rennet, B, purified calf rennet. Dependent axis represents relative apparent absorbance.

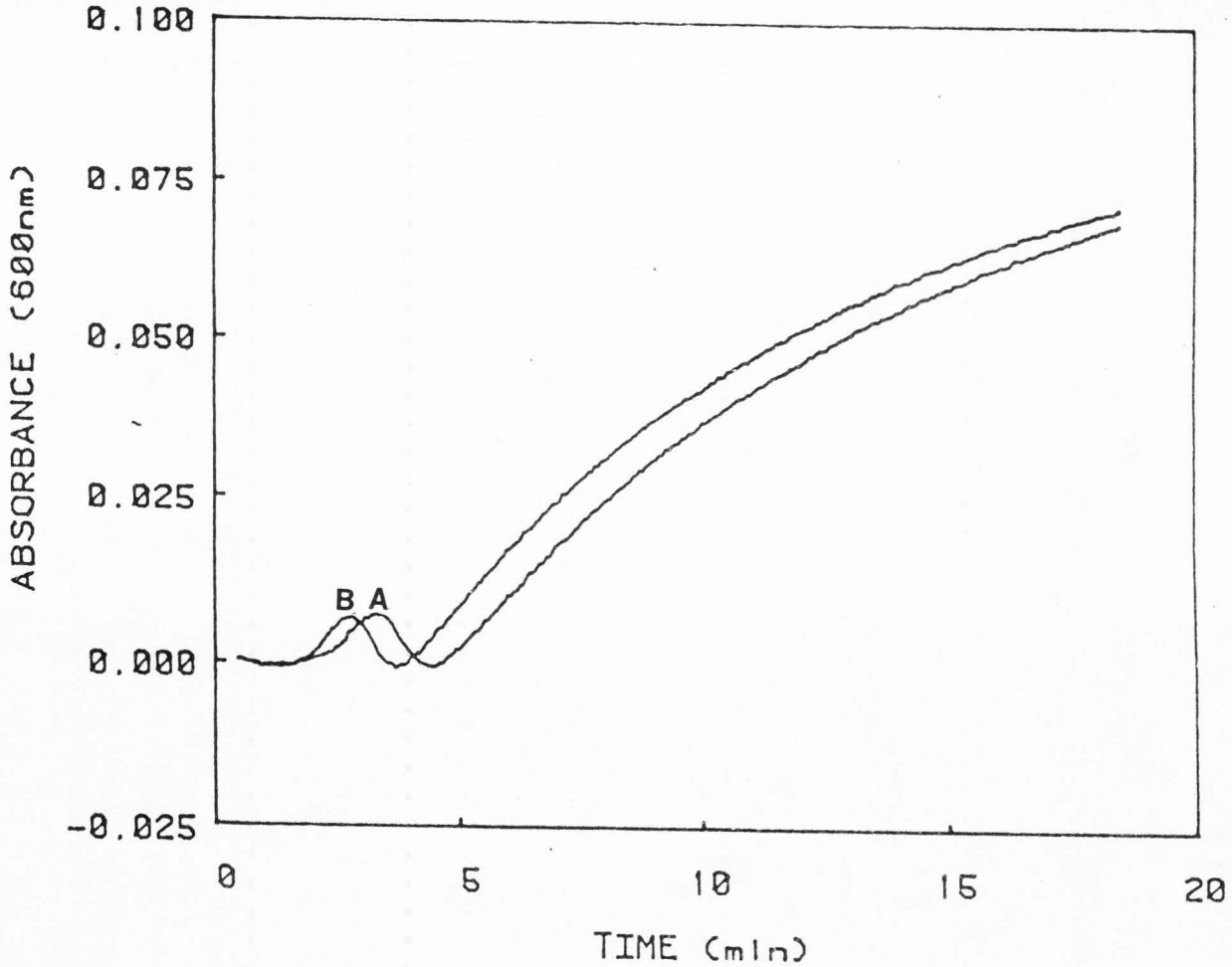

Figure 11. Coagulation curves of commercial and purified bovine rennet. A, commercial, B, purified. Dependent axis represents relative apparent absorbance.

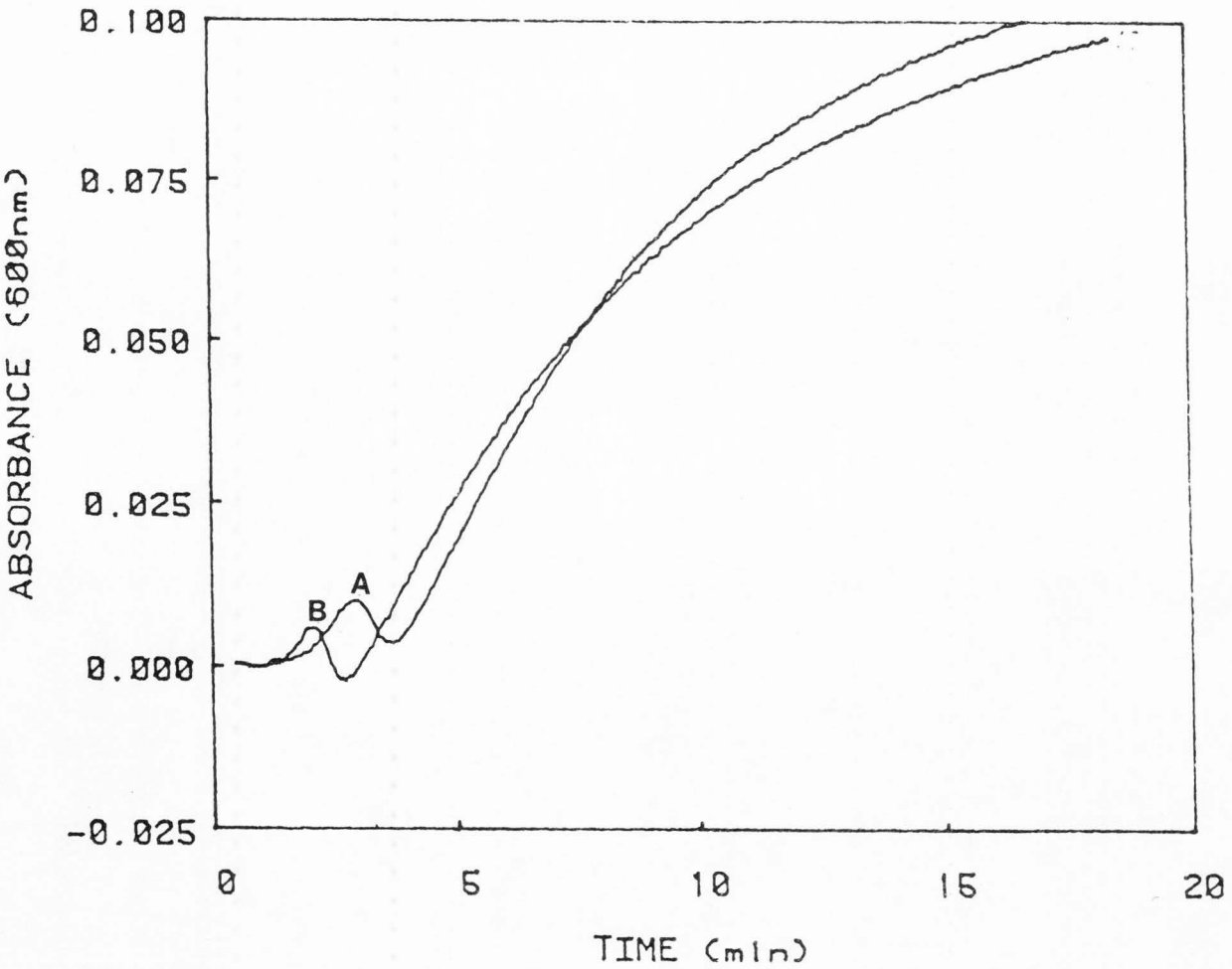

Figure 12. Coagulation curves of commercial and purified calf rennet/ porcine pepsin mixture. A, commercial, B, purified. Dependent axis represents relative apparent absorbance.

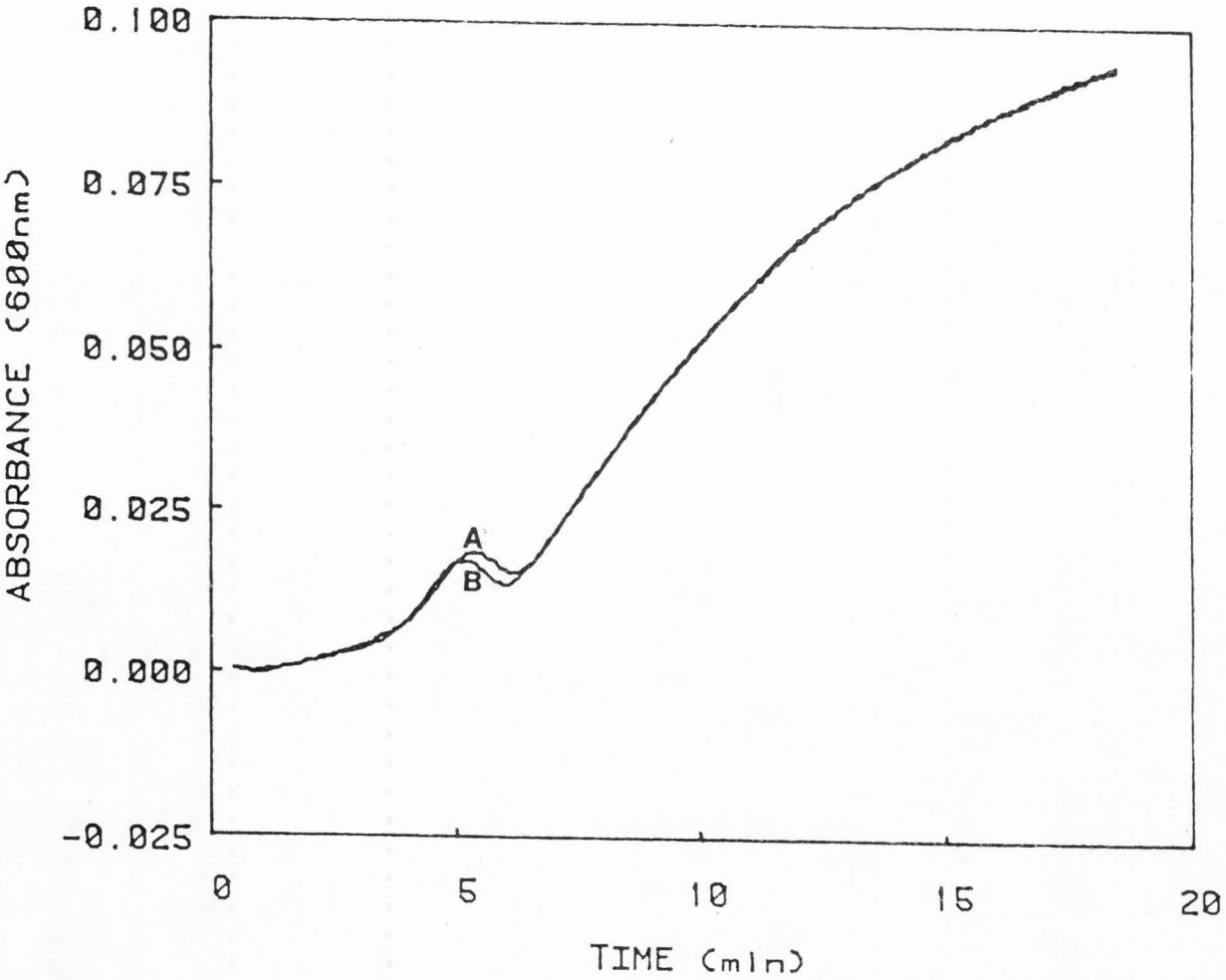

Figure 13. Coagulation curves of commercial and purified M. miehei protease. A, commercial protease, B, purified protease. Dependent axis represents relative apparent absorbance.

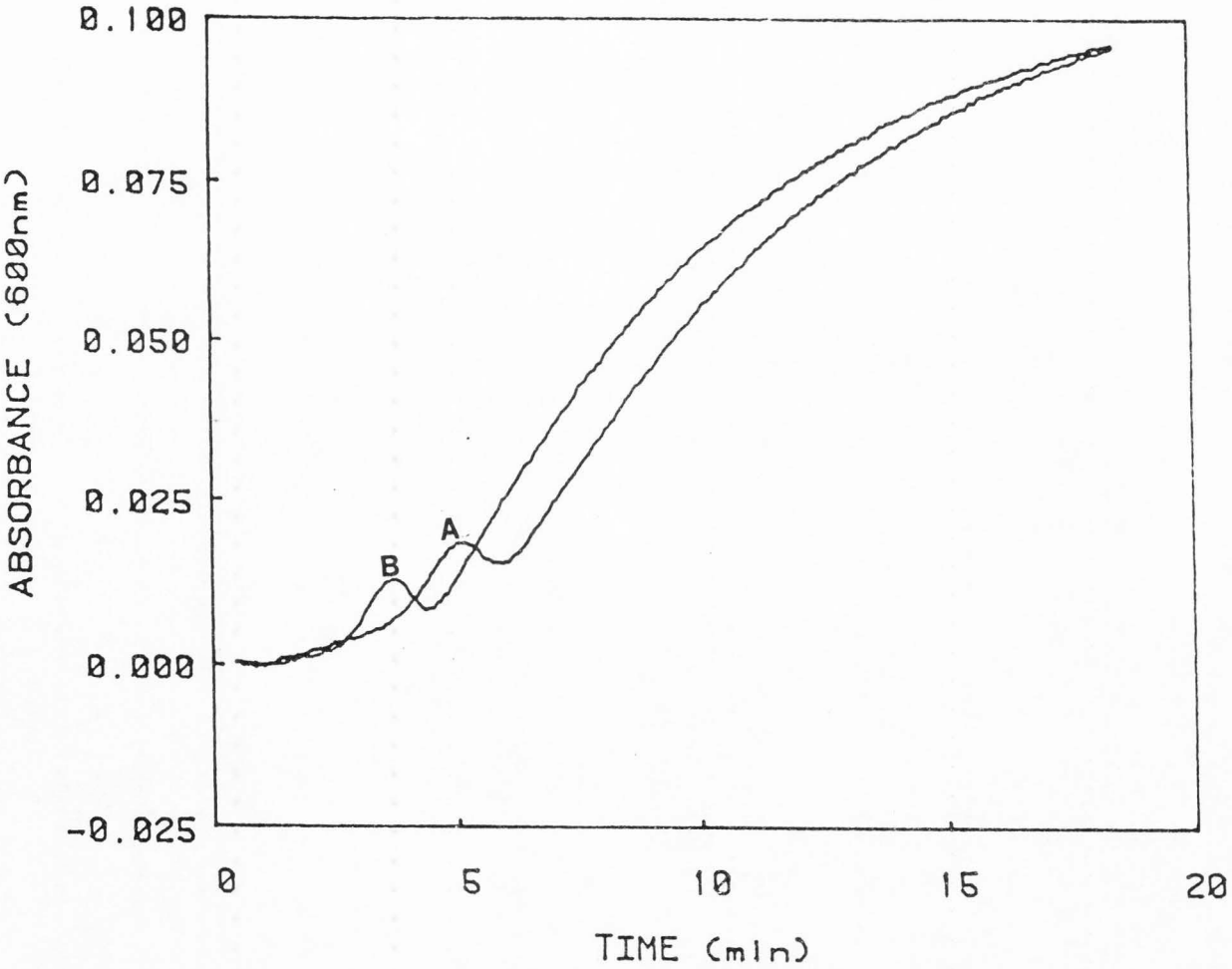

Figure 14. Coagulation curves of commercial and purified modified M. miehei protease. A, commercial protease, B, purified protease. Dependent axis represents relative apparent absorbance.

DISCUSSION

Protein Recovery

Percent protein lost to whey from five different commercial milk clotting enzyme preparations: calf rennet, bovine rennet, calf rennet/porcine pepsin mixture, M. miehei protease and modified M. miehei protease (Figure 2) were significantly different. Calf rennet resulted in more protein retained in curd than other enzymes. The mean value for protein lost to whey from this enzyme is 1.01 and the standard error of mean is .0174. Both bovine rennet and modified M. miehei resulted in lower protein lost to whey than calf rennet/porcine pepsin mixture and M. miehei. The mean value for protein lost to whey from bovine rennet was 1.05 and the standard error of mean was .021. Likewise the mean and standard error of mean values for modified M. miehei were 1.0363 and .020 respectively. The calf rennet/porcine pepsin mixture and M. miehei protease showed higher protein lost to whey than the others. The mean value for these two enzymes were 1.1132 and 1.1135 respectively, and the standard errors of means for these enzyme were .019 and .022 respectively. Calf rennet/porcine pepsin mixture and M. miehei protease were not significantly different from each other and the bovine rennet, calf rennet and modified M. miehei protease were not significantly different from each other. However, the two groups were significantly different from each other.

Figures 3 through 7 represent protein lost to whey from each pair of commercial enzyme and improved enzyme. Reduction in protein

lost to whey after purification was observed in all five pairs, but some differences are significant and some are not. Improved bovine rennet (Figure 4) and calf rennet/porcine pepsin mixture (Figure 5) showed significant reduction in protein lost to whey as compared to commercial samples. This agrees with previous work in finding that gel permeation decreased proteolysis by bovine rennet and calf rennet/porcine pepsin mixture more than the other three enzymes (Shaker and Brown, 1984). This suggests that reduction in percent protein lost to whey from improved enzyme could be due to removal of those fractions which have high proteolytic activity and low clotting activity or no clotting activity. Excluding these fraction increases enzyme specificity for milk clotting. Ultimately, this should result in less bitter flavor in ripened cheese. (The means and standard errors of means for the paired commercial and improved enzymes are given in the Appendix.)

Calf rennet, M. miehei and modified M. miehei proteases (Figures 1, 2 and 3) each showed no significant difference in protein lost to whey from the pair of commercial and improved preparations. The fractions with high clotting activity could also have high proteolytic activity. It is not possible to reduce such proteolysis of the clotting fraction by size exclusion separation.

The primary cleavage site for the clotting reaction is the Phe_{105}-Met_{106} bond of κ-casein. Proteolytic attack at other sites in κ-casein, or on α_s or β-caseins, is generally undesirable because it causes casein degradation and eventually increases percent of protein lost to whey. Chymosin is known to give minimum general

proteolysis, and substitutes are usually chosen on the basis of high clotting activity (rapid attack on the Phe_{105}-Met_{106} bond) of -casein combined with low general proteolytic activity. High general proteolysis may lead to losses of peptide material into solution during the curd-forming process, resulting in decreased yield of curd. The attack of α_s and β-caseins by chymosin is slower than the specific attack on κ-casein by a factor of about 100, so that such reactions occur to only a minimal extent during curd formation (Dalgelish, 1982).

Acid proteases other than chymosin show different rates of non-clotting proteolysis and prefer different sites in α_s, and β-caseins. Porcine pepsin is considerably more proteolytic than chymosin (De Koning, et al., 1978). Acid proteases are less restricted in their action toward the Phe_{105}-Met_{106} bond of κ-casein than is chymosin. Chymosin shows less non-specific proteolysis, and proteolysis of caseins other than κ-casein is relatively slow in relation to the primary attack on κ-casein.

Coagulation Properties

Coagulation by the five commercial and improved enzyme preparations was followed by monitoring of apparent absorbance of Berridge substrate at 600nm. Figure 8 shows the coagulation curves for the five commercial enzyme preparations after adjusting their activities to the same activity. Measurements were in absorbance units, and are a consequence of light scattering changes caused by changes in molecular weight, size, and number of colloidal casein micelle aggregates.

As a result of enzyme action on κ-casein, the macropeptide part of κ-casein is released (Payens, 1978). This part represents 10-15% of the weight of whole casein, therefore, a decrease in the molecular weight of about 3-4% of the micelle is expected by the time all of the κ-casein is hydrolyzed (Payens, 1977). Also, the average diffusion coefficient of the micelles shows a decrease (Payens, 1977). Decrease in the average molecular weight, reduction of volume and change of shape of the casein micelles are required to cause the decreases in apparent absorbance where the shoulders appear on the curves in Figure 8 (Walstra, 1979). After that, apparent absorbance starts to increase. Time from enzyme addition until visually observable coagulation is believed to be attributed to time required for the enzyme to produce an appreciable amount of aggregable material and the time required for this material to aggregate (Overbeek, 1952 and Payens, 1978). As κ-casein is hydrolysed, the micelles gradually become less stable, leading to an increase of aggregation rate (Darling and Van Hooydonk, 1981). After formation of the gel network, apparent absorbance continues to increase as gelation (coagulum firming) takes place (Garnot and Olson, 1982, and McMahon and Brown, 1982). Darling and Van Hooydonk (1981) reported that the first visible signs of coagulation occur when particles of the order 10 to 20 μm are produced, which is presumed to happen when the average particle diameter is approximately 1 μm.

In Figures 8 to 14 there is a noticeable shoulder in apparent absorbance versus time curves. These shoulders represent some changes of the aggregation process before visual coagulation.

McMahon, et al. (1984) attributed the appearance of these shoulders in apparent absorbance measurement to transformation of the milk from a system of essentially independent clusters to one in which the whole volume is filled by a network. They also thought that the reduction of rate of increase of apparent absorbance is due to change of average molecular weight and z-average particle scattering factors as the coagulation criticle point is passed. The coagulation criticle point is a point at which the aggregating particle mean free distance is so reduced that an explosive growth rate in particle size takes place. After this critical point has been reached the majority of particle collisions result in addition to the main floc. Goodarz-nia (1978) reported that these particle chains bend and rearrange themselves, after they come into contact, to form more stable positions. The coagulation system changes from one of independent aggregating particles to an extended network composed of interconnected casein micelle chains, and the milk ceases to behave as a true fluid of constant viscosity (Tuszynski, et al. 1968). Hardy, et al. (1981) found a similar shoulder in the coagulation curve when he used reflection photometry.

An increase in apparent absorbance continues as the coagulum solidifies and casein "free" at the coagulation point becomes associated with the space network. Dalgleish (1981) reported that at initial coagulation, 11% of the casein is not incorporated into the coagulum network. The incorporation of this "free" casein into the gel network, as well as changes in the structure of the network chains, should be considered in any explanations of the gelation

process (Storry and Ford, 1982). In coagulum consolidation by strand formation, milk serum would be entrapped by the space network and gathered into "microdroplets" between strands (McMahon, et al. 1984). Effects of internal "micro syneresis" were observed as an eventual decrease in apparent absorbance.

The five commercial milk clotting enzyme preparation showed different coagulation time and rates of curd firmness (Figures 8 and 9). The coagulation time for bovine rennet and calf rennet/porcine pepsin mixture are shorter than those for calf rennet, M. miehei and modified M. miehei. However, the rate of curd firmness of bovine of calf rennet/porcine pepsin mixture are crossed over with that of calf rennet after about 5 min of enzyme addition. As we mentioned earlier, that the curd firmness in coagulation curves is represented by the increase in apparent absorbance after the coagulation shoulder. The increase in apparent absorbance is thought to be a result of coagulum consolidation and incorporation of free casein into coagulum.

The microbial enzymes from M. miehei and modified M. miehei showed lower rates of curd firmness and also longer coagulation times. This could be attributed to the high non-specific proteolysis by these proteases. Yun, et al. (1981) reported that rate of increase of curd firmness decreases as the extent of κ-casein proteolysis increases. They found that rate of curd firmness of calf rennet > pepsin > M. miehei and M. pusillus proteinases > papain > trypsin. It has been reported that phosphoserin group of β-casein play an important role in curd

firming (Yun, et al. 1982). Therefore, the slow increase in rate of curd firmness for non-specific proteolytic activity attributed to the tendency of these enzymes to remove the C-terminus (hydrophobic) portion of β-casein including the phosphoserine groups. This results in fragile curd and eventually slows rate of firmness (Yun, et al., 1982). Interaction of phosphoserine with Ca^{+2} is very important for curd consolidation. It is important to keep β-casein phosphoserine group in order to enhance the curd firming. The slow rate of curd firmness for M. miehei and modified M. miehei proteases may be a consequence of proteolysis of β-casein near the C-terminus.

The commercial and improved enzyme preparations were compared to determine if the fractionation affects coagulation time and initial curd firming rate. Purified calf rennet had a slower initial coagulation rate than the commercial preparation (Figure 10). The rate of curd firmness of both enzyme preparations crossed over after 17 min. It is possible that casein incorporated after clotting time formed crosslinks between network chains and accelerate the curd firmness.

Figure 11 shows the coagulation time and the curd firming rate for commercial and purified bovine rennet. The purified enzyme coagulated milk faster and the rate of curd firmness was faster. This could be because of the removal of non-specific proteolytic fractions which causes the proteolysis of β-casein and decrease the rate of firming.

Purified calf rennet/porcine pepsin mixture showed shorter clotting time and faster curd firming than the commercial enzyme. However, curd curves crossed 7 min or after enzyme addition. The increase of curd firming rate of purified enzyme in the first few minutes could be the removal of non-specific proteolytic fraction. Increase of curd firming rate of the commercial enzyme could be due the incorporation of free casein micelles in the gel net work after clotting time which is thought to increase the rate more than if all casein is incorporated at clotting time. The same explanation could be used for modified M. miehei protease (Figure 13).

M. miehei proteases showed no difference in clotting time and curd firmness before and after purification. This because we could not remove the non-specific proteolytic fraction by gel filtration.

SUMMARY

There were significant differences among commercial milk clotting enzymes in their ability to retain protein in curd and reduce the amount lost to whey. Percent protein lost to whey was used as the criterion in comparing the protein recovery in curd among these enzymes. Gel permeation using Sephadex G-100 affected the coagulation properties of these enzymes. Improved enzyme preparations from microbial sources showed no significant differences in their protein recovery in curd. Improved enzyme preparations from animal organs did show significant difference in their protein recovery in curd. Improved bovine rennet and calf rennet/porcine pepsin mixture yielded a noticeable reduction in percent protein lost to whey.

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APPENDIX

Table 1. Analysis of variance of protein lost in whey from five different commercial milk clotting enzyme preparations. Protein in whey is the dependent variable.

Source	df	Sum of squares	Mean square	F	α
Enzyme preparations	4	.1922	.04806	5.14	.0008
Error	110	1.0281	.00934		
Corrected total	114	1.2203			

Table 2. Duncan's multiple range test comparisons of the effect of enzyme type on percent protein lost in whey from five different commercial milk clotting enzyme preparations.

Number of observations	Enzyme preparations	Mean (% protein lost in whey)	Grouping*
23	Mucor miehei	1.1135	A
23	calf rennet/porcine pepsin mixture	1.1132	A
23	bovine rennet	1.0511	B
23	modified M. miehei	1.0363	B
23	calf rennet	1.0134	B

*Means with the same letter are not significantly different.

Table 3. Analysis of variance of effect of calf rennet fractionation on protein recovery in curd. Percent protein lost in whey is the dependent variable.

Source	df	Sum of square	Mean square	F	α
Enzyme	1	.0045	.0045	.71	.403
Error	38	.239	.0063		
Corrected total	39	.244			

Table 4. Analysis of variance of effect of bovine renent fractionation on protein lost in whey. Percent protein lost in whey is the dependent variable.

Source	df	Sum of square	Mean square	F	α
Enzyme	1	.02198	.02198	5.46	.0255
Error	34	.1369	.0040		
Corrected total	35	.1589			

Table 5. Analysis of variance of effect of calf rennet/porcine pepsin mixture fractionation on protein lost in whey. Percent protein lost in whey is the dependent variable.

Source	df	Sum of squares	Mean square	F	α
Enzyme	1	.04213	.04213	6.09	.018
Error	34	.23512	.00691		
Corrected total	35	.27725			

Table 6. Analysis of variance of effect of M. miehei protease fractionation on protein recovery in curd. Percent protein lost in whey is the dependent variable.

Source	df	Sum of squares	Mean square	F	α
Enzyme	1	.00288	.00288	.51	.48
Error	34	.1929	.00567		
Corrected total	35	.1959			

Table 7. Analysis of variance of effect of modified *M. miehei* protease fractionation on protein recovery in curd. Percent protein lost in whey is the dependent variable.

Source	df	Sum of squares	Mean square	F	α
Enzyme	1	.02426	.02426	3.97	.053
Error	38	.23237	.00611		
Corrected total	39	.2566			

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