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BIOCHEMICAL CHARACTERIZATION OF RENNIN FROM THERMOACTINOMYCES VULGARIS.

by
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BIOCHEMICAL CHARACTERIZATION OF RENNIN FROM THERMOACTINOMYCES VULGARIS

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ABSTRACT

An extracellular milk-coagulant from Thermoactinomyces vulgaris was partially-purified by reverse osmosis, acetone precipitation, freeze-drying, dialysis and CM-cellulose chromatography. Purification was approximately 112-fold over the crude extract. Under similar conditions with DEAE-cellulose column chromatography, the fold-purification was approximately 18.

Proteolytic activity was determined upon hemoglobin at pH 3 to 10 and upon casein at pH 5.5 to 10. The microbial rennet exhibited optimal activity on casein and hemoglobin around pH 7. Commercial calf rennet exhibited optimal activity at pH 3 on hemoglobin and no activity above pH 6 on either substrate.

For both enzymes, optimal milk-clotting activity was at pH 5.6 and clot formation at pH 7.0 was greatly delayed.

After a two hour exposure at ambient temperatures, the pH stability range was 5.6 to 11 for the microbial rennet and 3 to 7 for commercial calf rennet.

In solution, the microbial rennet was inactivated by exposure to 65°C for 5 minutes, and after 24 days at 25°C. The crude acetone microbial powder exhibited no loss in activity after one year at -10°C and in solution retained 90% of its original activity after 24 days at -10°C and 10°C.

The effects of NaCl, KCl, $CaCl_2$ and $BaCl_2$ on milk-clotting activity of the microbial rennet and of commercial calf rennet were influenced by the composition of the substrate (i.e. the presence or absence of $CaCl_2$ in the milk, the ratio of milk to buffer).

Calf rennet was more sensitive to $CaCl_2$ and $BaCl_2$ than the microbial rennet. Slight activation at low concentrations of

NaCl and KCl, however, was more evident for the microbial rennet than the calf rennet. Higher concentrations (0.3 M) of all the chloride salts reduced the activity of both enzymes.

The microbial rennet was strongly inhibited by low concentrations of KCN (0.7 mM) while commercial calf rennet was unaffected by concentrations as high as 0.7 M.

The electrophoretic patterns of the microbial rennet and calf rennet were compared by polyacrylamide disc gel electrophoresis at pH 6.2. Casein was incorporated into the gel to identify proteolytically-active bands. There was good correlation between protein bands and proteolytically-active ones. There were three active bands for each enzyme. However, only one active microbial band possessed a similar R_f value to an active calf rennet band. For calf rennet, no inactive protein bands were detected while for the microbial rennet there were several such bands.

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INTRODUCTION

Primitive cheese was a product of the natural souring of milk through lactic acid fermentation. Through the years as the status of cheese increased as a protein source, manufacturing methods improved. Today, cheese production is highly sophisticated and the conditions for it are highly defined. This is partially due to the use of vegetable and animal extracts to curdle the milk directly. The term rennet (rennin if pure) has been universally accepted to indicate any enzyme preparation yielding a relatively stable curd (Sardinas, 1972).

In Canada, cheese production increased from 131 million pounds in 1962 to 249 million pounds in 1972. For 100 pounds of cheese, 3 ounces of commercial calf rennet are necessary.

The most commonly-used and commercially-available rennet is obtained from the fourth stomach of unweaned calves. In Canada from 1962 to 1972 the percentage slaughter of calves has remained relatively constant (approximately 80%). However, the estimated farm output of calves has decreased drastically during the same period - 1332 x 1000 in 1962 to 801.1 x 1000 in 1972. This is mainly due to high production costs and the low market prices for cattle even though the demand is great. So a problem of acquiring sufficient rennet is developing. Other countries are also faced with this problem. In certain countries like Israel and India there is another dimension to the problem. Due to religious convictions, cheese made from calf rennet is unacceptable. Therefore during the past few years the search for a suitable rennet substitute has been intensified. The three avenues being explored are plants, animals and microorganisms.

Certain criteria for rennet substitutes have been proposed (Babbar et al., 1965; Sardinas, 1969). There should be a

satisfactory yield of cheese with no increase in loss of either fat or protein as compared to the production of cheese from calf rennet. The cheese should have the desired textural, physical and organoleptic properties. There should be no radical change in the standard cheese manufacturing procedure unless it represents a distinct advantage to the producer. Milk should be coagulated in the temperature range of 25°C to 45°C and a pH range of 5 to 7. The enzyme preparation itself should be water-soluble, have acceptable color and odor, be non-toxic, be free of antibiotic activity and not possess excessive proteolytic activity. It should be contaminated only with a minimal level of undesirable enzymes and be free of pathogens or undue numbers of micro-organisms.

Gupta & Pereira (1974) reported the satisfactory production of Canadian cheddar cheese on a laboratory scale using a rennet isolated from Thermoactinomyces vulgaris.

In this study, different methods of purifying the milk-coagulant from T. vulgaris were examined in addition to a determination of the effects of certain conditions (pH, temperature, metals) on milk-clotting activity. For the majority of the latter studies, similar trials were carried out with commercial calf rennet. Also a comparison based on proteolytic activity was undertaken using polyacrylamide gel electrophoresis.

LITERATURE REVIEW

Higher plant Rennets

Fourteen genera of plants have been reported to produce milk-curdling enzymes. The enzymes are not restricted to one specific plant organ but have been isolated from leaves, latex, roots, sap, buds, flowers, fruit and seeds. The majority are, however, too proteolytic to be useful for cheese manufacture (Babbar et al., 1965; Sardinas, 1969, 1972).

The two most intensively-studied enzymes are papain from Carica papaya and ficin from the genus Ficus, specifically F. glomarata, F. religiosa and F. carica. Cheese from papain is bitter and considerably lipolysed while from ficin there is a low yield of curd and the cheese is also bitter compared to cheese from calf rennet. The difference may be due to their mode of activity. For calf rennin, serine as well as histidine and lysine residues have been reported at the active site (Babbar et al., 1965; Yu et al., 1971). For ficin and papain, a sulfhydryl group is essential for activity (Babbar et al., 1965).

From the Palestinian F. carica, two coagulating fractions have been reported (Zuckerman-Stark & Leibowitz, 1961, 1963). One is a proteolytic fraction which causes coagulation and subsequent hydrolysis of the clot and the second is a milk-curdling fraction with little proteolytic activity. A stable cheese (cheese held for 14 days at 4-7^{o}C) was produced with the latter.

Extracts from the berries of Withania coagulans had shown great potential with initial reports of acceptable cheese. However, in subsequent work all types of cheese produced developed a bitter taste (Sardinas, 1969).

Cheese comparable in consistency and taste to cheese from calf rennet has been produced with an enzyme preparation from the

fruit of Cucurbita pepo (pumpkin) (Berkowitz-Hundert et al., 1963, 1964).

Blossoms of Cynara cardunculus (prickly artichoke) yielded an extract with which a satisfactory Camembert cheese could be produced, but Serpa cheese was unacceptable (Sardinas, 1969).

Another seemingly favorable source is an extract from Benincasa cerifera (ash gourd) (Ramamurtia & Johar, 1964). The preparation clots cow and buffalo milk as well as peanut "milk". Subsequent proteolysis is negligible. However, no cheese trials have been reported.

Although plant rennets have been very well investigated, none are commercially available.

Animal Rennets

Animal rennets have been isolated from sheep, goats, swine, chickens, rabbits and buffaloes, besides from calves of the domesticated cow (Sardinas, 1969). The source again is expensive and the cheese not entirely satisfactory. This is also true for cheese from other animal proteases (chymotrypsin, trypsin and pepsin).

Pepsin produces a better cheese than the other enzymes and has been subject to more than a cursory evaluation. At one time it was considered to be identical to calf rennin but it has been found to be similar only in some respects. For example, in the hydrolysis of peptide bonds of oxidized B-chain of insulin, both enzymes hydrolyse the same bonds except pepsin also hydrolyzes three additional bonds (Whitaker, 1972).

The major disadvantages of using 100% pepsin are - a longer

setting period is required; the clot is not as firm; there is a greater loss of fat and a bitter flavor develops during ripening.

Today a 50/50 mixture of rennet and pepsin is popular in producing many types of cheese including long-hold cheddar. In Canada 80% of the customers of a major rennet supplier use this blend (Lally, 1974). Although the cheeses are inferior to those made with 100% calf rennet, they are acceptable. Another added advantage is that the blend is less expensive than rennet due to the low cost of pepsin. The ratio can also be manipulated to suit the manufacturer's individual requirements (Sardinas, 1969).

There is also a report describing the production of a suitable type of cheddar cheese with various animal and vegetable enzyme preparations whose proteolytic potencies were partially inactivated by an assortment of treatments (Ilany-Feigenbaum & Netzler, 1969). The activity of trypsin and pancreatin was reduced by X-ray irradiation while that of ficin, papain, and bromelain was reduced by the inhibition effect of monoiodoacetic acid or sorbic acid. The proteolytic potency of pepsin was reduced by raising the pH to 6.6.

Microbial Rennets

Micro-organisms offer the widest area of exploration for suitable rennet substitutes. Their growth can be closely controlled so enzymes exhibiting the desired activity, substrate specificity and mode of action can be generated. Also they can be produced economically on any desired scale (Babbar et al., 1965; Sardinas, 1969). It is in this area that the search for a suitable rennet substitute has been predominant and the greatest advances made.

The idea of exploring micro-organisms originated with the individual research of Conn and of Gorini in 1892 (Sardinas, 1972).

The scope or vastness of this area has been pointed out by Sardinas (1972). Approximately two thousand bacteria and fungi have been evaluated. Forty-three bacterial genera (less than 7% of all genera) have been reported to produce milk coagulants while thirty-eight fungal genera (less than 1% of all known genera) have been reported.

Greater progress has been made in the area of fungal rennets than bacterial rennets. Sufficiently suitable enzymes are being produced commercially from three fungal species - Endothia parasitica, Mucor miehei and M. pusillus. Cheese is satisfactory but a better quality is possible when they are used in combination with calf rennet. These rennets are marketed in Australia, Europe, Japan, Latin America, the United Kingdom and the United States. In Canada, none have been approved, nor will be approved, until the cheese produced using the fungal rennet is graded (Lally, 1974).

Other fungal rennets have been patented but are not yet produced commercially. Some of the species involved are Basidiobolus ranarum, Colletotrichum atramentarium, Conidiobolus brefeldianus, C. villosus, Coriolus consors, C. versicolor, C. hirsutus, Entomophthora apiculata, E. coronata, Eurotium oryzae, Ascochyta visa, Fomitopsis rosea, F. annosa, F. pinicola, Irpex lacteus, Monascus anka and Rhizopus niveus.

For bacterial rennets four species are predominant - Bacillus polymyxa, B. mesentericus, B. cereus and B. subtilis. Patents have been issued for these as well as for B. brevis, B. mycoides, and B. fusiformis. As with the fungal rennets, better-quality

cheese is obtained when they are used in conjunction with calf rennet. None are produced commercially.

The properties of the more important bacteria and fungi listed above have been summarized by Sardinas (1969, 1972).

Adaptation of the cheese-making process to make the best use of the milk-curdling properties of these enzymes is currently another area of research.

Tendler & Burkholder (1961) first reported the production of an exoenzyme with rennin-like activity from Thermoactinomyces strains. This genus belongs to the order Actinomycetales. Although true bacteria, Actinomycetales are mold-like (filamentous) in appearance. The Streptomycetceae family develop a true mycelium, either forming conidia (spores) in chains at the end of the hyphae or bearing single conidia on short conidiophores. Species of Thermoactinomyces are actinomycetes of the latter type which are favored by high temperatures. Isolates, however, can be grown at temperatures ranging from 30°C to 67°C depending on the interaction of components in the nutritional environment. They do not reduce nitrates nor grow on media lacking an organic source (reduced form) of nitrogen (Tendler & Burkholder, 1961).

After several Thermoactinomyces species were examined for rennin activity, T. vulgaris was selected as the most promising source of a milk-coagulant (Gupta & Pereira, 1974). Maximal enzyme production was possible after 35 hours' growth in a trypticase soy broth media fortified with glucose at pH 5.6, at 37°C, with an aeration rate of approximately 2 ml. air / ml. medium / minute. Laboratory batches of Canadian cheddar cheese produced with a crude acetone powder of the enzyme were comparable to

batches produced with commercial calf rennet. Both were evaluated after holding at 7°C for 9 to 14 weeks. Further cheese trials, involving longer ripening periods, are being carried out at the present time.

Purification

The specific function of rennets in intact micro-organisms is not known but has been associated with spore formation (Osman et al., 1969).

As the enzymes tend to be extracellular, there is an initial problem of concentrating the fermentation liquor after it has been separated from the cells.

Separation is accomplished by centrifugation and/or by filtration (Srinivasan et al., 1962; Chu et al., 1973; Farr et al., 1974).

Techniques for the initial reduction of volume have involved concentration under vacuum at temperatures below 40°C (Sardinas, 1968), slowly freezing the supernatant at -10°C so the protein is concentrated at the top in a narrow band (Farr et al., 1974), reverse osmosis (Gupta & Pereira, 1974) and precipitation with ammonium sulphate, ethanol, methanol, isopropanol, acetone or tannin (Osman et al., 1969; Kawai, 1970). In handling large volumes, precipitation with solvents at this stage can be expensive.

Further purification depends on the properties of the enzyme and on the discretion of the researchers. The schemes usually involve a combination of precipitation with ammonium sulphate or organic solvents, ion-exchange resins and gel filtration (Arima, 1971; Kawai, 1971; Sternberg 1971; Farr et al., 1974). Filtration through activated carbon (Sardinas, 1968) and the absorption

of undesirable proteolytic enzymes on silicate (Moelker & Majjhijsen, 1972) have also been used. Crystallization is possible either by evaporation at 3-4°C requiring up to two months or by dialysis requiring only a week (Arima, 1971).

Electrophoresis

Generally the enzymatic digestion of milk or specific casein fractions as followed by electrophoresis is one basis of comparing the action of rennets from various sources (plant, animal and microbial). Polyacrylamide, both vertical (Fox, 1968) and disc (Puhan, 1971; Kawai, 1971; Kovacs-Prost & Sanner, 1973) electrophoresis, as well as starch gel electrophoresis (Itoh & Thomasow, 1971) and cellulose acetate electrophoresis (Sternberg, 1971) have been used successfully for this purpose.

In independent studies, Cserhati (1970) and Vamos & Morvai-Racz (1970) examined various microbial and animal rennets by paper electrophoresis. They concluded that the quality of the electrophoretograms and the recovery of milk-clotting and proteolytic activities depended on the type of buffer as well as on its pH and ionic strength and on the degree of purity of the enzyme.

By incorporating casein in the polyacrylamide gel, Asato & Rand (1971) were able to identify proteolytically-active bands after vertical gel electrophoresis of rennin and prorennin at pH 7.1. After incubation in 0.3 M phosphate buffer, pH 2.0, at 37°C for 1 hour and staining with 1% amido black solution, the active bands appeared as clear areas in a blue background.

Shovers & Possum (1972) identified active microbial and animal electrophoretic fractions after vertical polyacrylamide gel electrophoresis at pH 4.5 by layering milk over the flat gel

slab. In active zones of milk-clotting activity, the milk appeared as filamentous strands embedded in the gel.

Garnot et al. (1972) employed a 1% casein agar gel to determine active fractions of calf rennin and bovine pepsin after agarose acrylamide gel electrophoresis at pH 5.4. The agarose gel was layered on the casein gel and the two incubated at 37°C in a humid atmosphere to allow the enzymes to diffuse into the casein gel. The casein gel was then stained with Coomassie Brilliant Blue. The active enzymatic bands appeared as white spots.

Using disc polyacrylamide gel electrophoresis according to Davis (1964) at pH 8.3, employing tris buffer, Vamos-Vigyazo et al. (1973) were able to demonstrate the heterogeneity of various animal and microbial rennets, The isoenzyme pattern obtained also depended on the purity of the preparation. No attempt was made to recover milk-clotting and proteolytic activities from the electrophoretically separated proteins as the activities of the majority of the preparations were inactivated in tris buffer at pH 8.3.

The electrophoretic pattern obtained by isoelectric focusing has also been suggested as a means of characterizing different types of rennet (de Koning & Draaisma, 1973).

As long as the conditions are clearly defined, electrophoresis is a valuable tool for following purification and for comparing rennet preparations. The effects of pH, buffer, temperature et cetera must be considered in establishing an electrophoretic procedure and interpreting results.

Rennet Activity

The coagulation of milk consists of a minimum of three distinct

phases. Firstly, there is enzymatic hydrolysis of one or more specific peptide bonds in κ casein. This action destroys the micelle-stabilizing power of the κ casein fraction so that in the presence of calcium ions the casein coagulates. The third phase is the slow continuous enzymatic hydrolysis of all the casein components or general proteolysis. With excessive proteolysis, bitter polypeptides are released which adversely affect the organoleptic properties of the cheese. Therefore only enzymes with high milk-clotting activity and low proteolytic activity are suitable for cheese production (Tam & Whitaker, 1972).

Sardinas (1972) reviewed the various methods of determining milk-clotting and proteolytic activities.

Milk-clotting activity is determined by measuring the time required to clot a suitable milk substrate under specified conditions. It is not a velocity measurement. For example, the Soxhlet unit is defined as the amount of enzyme which will clot one ml of milk in forty minutes (Sternberg, 1971) while the Berridge unit is the amount of enzyme which clots 10 ml of milk in 10 minutes (Osman et al., 1969) and the Ernstron unit is the amount which clots 1 ml of milk in 1 minute (Chu et al., 1973). Conditions of assay such as the nature of the substrate, pH and temperature must be specified. A thromboelastographic measurement was found to be satisfactory for measuring clotting time (de Man & Batra, 1964). No unit for milk-clotting activity was defined.

The determination of proteolytic activity by a non-velocity measurement is also common practice. For example, activity has been defined as the amount of enzyme which produces an increase in absorbance of one in thirty minutes (Farr et al., 1974) or the amount of enzyme which releases the equivalent of one μg

tyrosine for conditions of assay (Sternberg, 1971). Proteolytic activity has also been expressed directly as absorbance at 660 nm (Arima, 1971). The splitting of a -Phe(NO_2)-Nle- bond in a synthetic hexapeptide has also been proposed as a means of determining proteolytic activity of rennets (Raymond et al., 1973). The presence of other proteolytic enzymes could be detected by comparison with a clotting test.

The lack of established universally-acceptable methods for determining milk-clotting and proteolytic activities is a major difficulty in comparing work on different rennets, especially as the number increases. Usually in published results for a new rennet (effects of pH, of temperature, of metals and reagents et cetera on clotting activity), results obtained under the same conditions with calf rennin are also stated (Tsugo & Yamauchi, 1959; Sardinas, 1968; Pozzar-Hajnal et al., 1970; Kawai, 1970). In studying the effect of metals and reagents, the compounds maybe either added directly to the substrate (Kawai, 1970) or pre-incubated with the enzyme at specified conditions before determining activity (Arima, 1971; Farr et al., 1974).

The initial rates of hydrolysis and extent of hydrolysis after 1,440 minutes of whole, α, β and κ casein by crystallized rennin, crystallized pepsin, a purified Mucor pusillus rennet and a purified Endothia parasitica rennet at pH values from 3 to 6 have been compared (Tam & Whitaker, 1972). The patterns obtained depended on the substrate, pH and enzyme.

The kinetics of calf rennin on whole milk and casein prepared by ultracentrifugation (Castle & Wheelock, 1972, 1973) and of a purified Mucor pusillus rennet on κ casein (Sanner & Kovacs-Proszt, 1973) have been investigated. The described methods, however, require long time periods or special equipment.

The action of calf rennin on whole milk was determined by measuring the rate of release of glycopeptides at 217 nm. Its action on casein was determined by following the increase in turbidity of the casein solution at 600 nm. Dialysis for two weeks was necessary before the absorbance at 217 nm could be measured as trichloroacetic acid used to stop the reaction at predetermined intervals interfered with this absorption. At 600 nm, it was impossible to record the action of rennin without a kinetic box.

For the Mucor pusillus rennet, stopped-flow spectroscopy was used.

Both parties concluded that the observed Km represented an average Km value for the different peptide bonds involved. Castle & Wheelock (1972, 1973) also suggested that the significant differences in Km and V among milk from different sources (cows) were due to differences in the ionic environment and in the structure and composition of the casein micelles especially the carbohydrate content of χ casein.

Due to the complexity of rennin activity as illustrated in the above kinetic studies, the indirect methods of determining milk-clotting and proteolytic activities as well as cheese trials will continue to be used in evaluating new rennets.

The physico-chemical properties and electrophoretic pattern of the rennet from T. vulgaris in comparison to calf rennet have not been thoroughly investigated. Also no purification scheme other than the one described by Gupta & Pereira (1974) has been proposed. A nine-fold purification was reported. Steps included centrifugation, concentration by reverse osmosis, acetone precipitation and dialysis, ultrafiltration, and freeze-drying.

MATERIALS & METHODS

Materials

Microbial rennet was isolated and purified from Thermoactinomyces vulgaris, ATCC 15733, obtained from the American Type Culture Collection, Washington, D.C.. Hansen's cheese rennet (standard) from the Horan-Lally Company Limited, Rexdale, Ontario, served as the commercial calf rennet standard. Homogenized pasteurized milk was obtained from the University of Manitoba Dairy. Casein (purified) was obtained from Matheson, Coleman & Bell, Norwood, Ohio and hemoglobin (Bovine Powder Type II) was purchased from the Sigma Chemical Company, St. Louis, Missouri. Carboxymethyl-cellulose (fine mesh, 0.67 meq exchange capacity / gram, lot 46B1070) and diethylaminoethyl-cellulose (fine mesh, 0.89 meq exchange capacity / gram, lot 122C2620) were products of the Sigma Chemical Company. Other chemicals and reagents were analytical grade.

Laboratory Methods

a) Rennet Activity Determination

The method employed was similar to that described by de Man & Batra (1964) except that a Mechrolab Clot Timer Model 202 A from Heller Laboratories was used and 0.1 ml enzyme was added to the substrate instead of 0.2 ml. In both studies the temperature of determination (37°C) and the amount of substrate (0.2 ml) were similar.

Milk-clotting units (MCU/ml) were calculated by the following formula -

$$MCU/ml = (1000 / T) \times \text{dilution coefficient}$$

where T equals clotting time in seconds.

The composition of the substrates employed are outlined in Table 1. Unless otherwise stated, substrate 1 was used.

b) Protein Determination

Protein was determined by the method of Lowry et al. (1951). Reagents were modified according to Miller (1959). Commercial Folin-Ciocalteu reagent was diluted ten times immediately before use. Crystalline bovine serum albumin (British Drug Houses, fraction V) served as the standard.

c) Proteolytic Activity Determination

The method of Sternberg (1971) was generally followed for preparing the substrates and for determining activity.

The substrates were 1% urea-denatured hemoglobin and 1% casein.

One gram of hemoglobin was dissolved in 30 ml water containing 18 grams of urea and 2 ml of 3 N NaOH. The solution was adjusted to the pH of determination and the volume increased to 50 ml. It was then diluted to 100 ml with 0.2 M citrate-phosphate buffer for pH 3 to 7 and 0.2 M boric acid - borax buffer for pH 8 and 9.

The casein was prepared by dissolving 1 gram of casein in 30 ml water to which 0.2 ml of 3 N NaOH was added and continually stirring over medium heat until dissolved. After cooling the volume was adjusted to 50 ml. The casein solution was then mixed with an equal volume of 0.2 M phosphate buffer for pH 6 to 8 and/or an equal volume of 0.2 M boric acid - borax buffer for pH 8 to 9.

Five milliliters of substrates were incubated with 1 ml diluted enzyme at 35.5°C for 20 minutes. The reaction was

Table 1 Composition of substrates

Substrate	
1	To 50 ml of milk containing 1 ml of 0.05 M $CaCl_2$, 2 ml of 0.01 M acetate buffer, pH 5.6, was added. The pH was then adjusted to 5.6 with 3 N lactic acid (Gupta, personal communication).
1A	same as # 1 but without $CaCl_2$
2	To 25 ml of milk containing 1 ml of 0.05 M $CaCl_2$, 25 ml of 0.1 M acetate buffer, pH 5.0, was added. Final pH was 5.6.
2A	same as # 2 but without $CaCl_2$
3	Same as # 2 but 25 ml of 0.1 M tris-maleate buffer, pH 5.0, was added instead of acetate buffer. Final pH was also 5.6.

stopped with 10 ml of 5% trichloroacetic acid. For a clear fast filtration, the mixture was kept at 60°C for 30 minutes, cooled in running tap water and filtered through Whatman # 42 filter paper. A blank made with 5 ml substrate, 10 ml trichloroacetic acid and 1 ml diluted enzyme was run with each assay under the same conditions. The absorbance of the clear filtrates was read at 280 nm against the blanks (Sternberg, 1971).

A standard curve of tyrosine (0 to 100 µg / ml water) was prepared by plotting the absorbance at 280 nm versus tyrosine concentration. Proteolytic activity was expressed as µg tyrosine produced / mg enzyme protein.

In determining the milk-clotting / proteolytic activity ratio (MCU / µg tyrosine) during the final purification steps, rennet activity was determined at pH 5.6 while the proteolytic activity was determined at pH 7 using casein.

Cultural Methods

a) Stock Cultures

Stock cultures were maintained on trypticase soy agar slants incubated for 24 to 36 hours at 37°C and stored at -10°C.

b) Preparation of Initial Inoculum

Growth from the above slants was transferred aseptically to a presterilized 250 ml flask containing 100 ml of trypticase soy broth (pH 5.6). The inoculated flask was placed on a gyratory shaker operating at 200 rpm at 37°C for 48 hours. This inoculum was then divided among four other presterilized 250 ml flasks, each containing 100 ml trypticase soy broth (pH 5.6). The flasks were incubated for another 48 hours

under the above conditions. This inoculum was used for one fermentation.

c) Cultivation and Harvesting

Cultivation was performed in a 14-liter Chemap p.e.c. fermentor using 8 liters of trypticase soy broth with 1% glucose (pH 5.6) (Gupta, personal communication). pH was maintained at 6.0 $\pm$ 0.4 with 6 N H_2SO_4 and 6 N NaOH. Compressed air was filtered through an activated carbon - glass wool filter at approximately 2 ml / ml medium / minute. Incubation proceeded at 37 ^{o} C for approximately 35 hours or until there was no significant increase in activity over a two hour period.

After each fermentation the broth was centrifuged using the Sorvall KSB continuous flow system at 25,000 x g. The exchange rate was approximately 700 ml / hour.

Purification

a) Reverse Osmosis

A Calgon Havens Osmotik Test System fitted with module 215 (molecular weight cut-off of 20,000) from Calgon Havens Systems, Pittsburgh, Pa. was used to reduce the volume of the supernatant by seventy-five per cent. The flow rate was approximately 25 ml effluent / hour at 400 psi. The concentration was performed at 10^{o}C.

b) Acetone Precipitation

Precipitation was carried out at 4^{o}C by mixing 3 volumes of concentrated supernatant with 7 volumes of cold acetone (0 to 0.7 fraction). The suspension was immediately centrifuged for

30 minutes at 12,000 x g at 0°C. The brown precipitate was resuspended in a minimal amount of 0.01 M tris-maleate buffer (pH 5.6) and freeze-dried. This product is subsequently termed the crude acetone microbial powder.

c) Ion-exchange

The DEAE-cellulose and CM-cellulose resins were precycled and packed according to the method outlined in the Whatman Advanced Ion-exchange Celluloses Laboratory Manual (W. & R. Balston Ltd., Maidstone, Kent, England). The final dimensions of the columns were 2.5 by 19.0 ± 0.5 cm.

Trials were carried out with both resins at pH 6.2 and 7.2 at 10°C using sodium chloride linear gradient elutions. The reservoir contained 500 ml of 0.01 M tris-maleate buffer and 0.5 M or 1.0 M NaCl, and the mixing chamber contained 500 ml of the same buffer but without NaCl. Flow rates varied from 25 to 30 ml / hour. Elution was monitored at 280 nm with an ISCO UA4 Absorbance Monitor. The eluate was collected on a time-controlled LKB 7000 Fraction Collector.

After each run the columns were washed with two liters of 1 M NaCl and then six liters of 0.01 M tris-maleate buffer at the desired pH and flow rate. Following the above procedure the same column could be used three times without full regeneration.

Sample preparation

0.7 grams of the crude acetone microbial powder was dissolved in 15 ml starting buffer and dialysed against 1 liter of the same buffer for 16 hours at 10°C. The amount added to the column depended on the protein assay, but was usually 5 to

10 ml containing 20 to 28 mg protein.

Polyacrylamide Gel Electrophoresis

a) Sample Preparation

Preliminary trials indicated that the best resolution could be attained by adjusting the protein concentration of the microbial rennet (after dialysis) and the commercial calf rennet to 2 mg protein / ml. One milliliter of diluted enzyme was mixed with 1 ml of 10% glucose, containing bromophenol blue which was used as a marker for the electrophoretic front. Fifty microliters of this was layered on the upper gel using a micro-syringe.

b) Electrophoretic Separation

Separation was carried out using the Savant disc electrophoresis apparatus with a Buchler power supply #31014A. The procedure outlined in the Polyanalyst Instruction Manual (Buchler Instruments Inc., Fort Lee, New Jersey) was generally followed.

The composition of the gel solution was adapted from Chen & Bushuk (1970) and is given in Table 2. The addition of casein for identifying rennin by the detection of proteolytic activity was recommended by Asato & Rand (1971). During runs involving casein, gels with casein but without rennet were run to check for possible casein migration. The buffer solution for the upper and lower electrode chambers was 0.01 M sodium phosphate buffer, pH 6.2.

The buffer, gels and samples were cooled to 10^{o}C, at which temperature electrophoresis was carried out.

Table 2 Composition of solutions for disc electrophoresis

(For electrophoretic runs without casein, 1 volume of 0.01 M phosphate buffer, pH 7.2 was added)

Chemicals	Upper gel, pH 6.2 per 100 ml 0.01 M phosphate buffer, pH 6.2	Volume ratio	Lower gel, pH 7.2 per 100 ml 0.01 M phosphate buffer, pH 7.2	Volume ratio
Solution A		2		2
Acrylamide, g	10.0		30.0	
N,N-methylene-bisacrylamide, g	0.8		0.8	
Solution B		1		1
N,N,N,N-tetra-methylenediamine, ml (pH adjusted to 6.2 or 7.2 with 3 N H_3PO_4)	0.12		0.32	
Solution C		2		4
Ammonium persulfate, mg	20		140	
Riboflavin, mg	4		-	
Solution D		-		1
Casein, g	-		0.8	

Electrophoresis was performed in two steps. An initial potential of 50 volts was applied for 30 minutes allowing the samples to concentrate in the upper gel, and then the voltage was increased to 100 volts to maintain the current as close as possible to 3 mA per tube. The total time involved was 100 minutes.

c) Staining

Detection of protein bands

The gels were rinsed in distilled water and then immersed in 0.02% Coomassie Brilliant Blue R250 in 10% trichloroacetic acid (TCA) for 24 hours. The gels were then destained with 10% TCA until the best possible resolution was attained (approximately 3 hours).

Detection of proteolytic activity

After electrophoresis the gels were incubated in 0.2 M phosphate buffer, pH 5.6 (200 ml / 4 gels) for 3 hours at 37°C. The gels were immediately stained for 5 minutes in 1% amido black solution containing acetic acid / methanol / water (1:5:5, v/v/v) and then washed in 5% acetic acid (v/v) in 50% methanol (v/v) overnight. The next morning, excess dye was removed by gently agitating the gel in 5% acetic acid. Active bands appeared as clear areas in a blue background of undigested casein (Asato & Rand, 1971).

Properties of the T. vulgaris rennet

a) Effect of Different Substrates on Milk-Clotting Activity

Various enzyme concentrations, expressed as µg or mg protein

/ ml were prepared with distilled water from the acetone microbial powder, dialysed residue, milk-clotting DEAE fraction and from commercial calf rennet. For the dialysed preparation, the acetone powder was dialysed against distilled water (pH 5.6) overnight. The substrates employed were numbers 1, 2 and 3 indicated in Table 1.

b) Effect of pH

Substrates for determining the optimum pH were prepared by mixing equal portions (20 ml) of milk and 0.05 M tris-maleate buffers of varying pH. 0.8 milliliters of 0.5 M $CaCl_2$ was added so that the final concentration of $CaCl_2$ was 0.01 M. The enzyme preparations studied included the dialysed residue, DEAE fraction and commercial calf rennet.

In determining the effect of pH on the stability of the above enzyme preparations, 0.2 M NaOH and 0.2 M HAc were used in adjusting the pH. The preparations were diluted so that the activity was directly proportional to enzyme concentration. Samples were allowed to stand 1 and/or 2 hours at ambient temperatures. The pH was readjusted to 5.6 and the residual activity determined. Substrates 1 and 2 given in Table 1 were used.

c) Effect of Temperature

For the heat stability study, the acetone powder was dialysed overnight against 0.05 M tris-maleate buffer, pH 5.6, and then diluted with same so that the activity was directly proportional to enzyme concentration. Aliquots of the enzyme were exposed to different temperatures for specific time periods and then cooled before determining the residual activity at 37°C. Substrates 1 and 2 given in Table 1 were

used.

Also, 5 ml aliquots of the above enzyme solution were stored at -10°C, 10°C and 25°C. Activity was determined over a one-month period.

d) Effect of Metal Ions and of Cyanide

Various concentrations of NaCl, KCl, $CaCl_2$, $BaCl_2$ and KCN were prepared using 0.1 M acetate buffer, pH 5.6. If necessary, the pH of the buffered solution was adjusted to 5.6. 1.5 milliliters of each solution was mixed with 0.5 ml enzyme. The tubes were incubated at 37°C for 10 minutes. A control containing 0.5 ml enzyme and 1.5 ml buffer was run with each assay.

The enzyme was prepared by dissolving a specific amount of acetone powder in buffer so that by mixing 0.5 ml of this and 1.5 ml buffer, the activity would be directly proportional to enzyme concentration. Substrates 1, 1A, 2 and 2A outlined in Table 1 were used.

RESULTS

Purification

Reverse osmosis proved to be a valuable means of initially concentrating the fermented broth as there was negligible loss of milk-clotting activity. More than 75% reduction of the volume was considered inappropriate when considering the capacity of the system. The machine tended to heat after continuous operation, even in the cool room. This in time had a detrimental effect on the enzyme. Shorter operating periods, however, were found to be time consuming.

Heat treatment at 55°C for 10 minutes and precipitation at pH 5 were found to be unsatisfactory. Neither contributed to further purification nor to volume reduction.

Concentration by ammonium sulphate precipitation and by acetone precipitation was attempted. As these treatments require dialysis, it was necessary to investigate the effect of different buffering systems and pH on activity. The best systems were shown to be tris-maleate and acetate (Table 3). Citrate had a detrimental effect on the enzyme. The enzyme was exceptionally stable from pH 5.6 to 7.5 in tris-maleate buffer. The experiments also indicated an approximately three-fold purification as a result of dialysis.

Attempts to precipitate the enzyme with ammonium sulphate were unsuccessful as the precipitate tended to remain suspended as a floc even after centrifugation. Results were inconsistent in terms of recovery and purification although the enzyme was usually present in the 0.4 to 0.7 range.

Acetone precipitation provided more consistent results. Trials indicated the presence of the enzyme in the 0.4 to 0.7 range (Table 4). For ease of handling, however, only one fraction

Table 3 Effect of dialysis by 0.1 M acetate buffer, pH 5.6; 0.05 M citrate buffer, pH 5.6; water adjusted to pH 5.6 with 6 N H_2SO_4; and 0.05 M tris-maleate buffer, pH 5.6 to 7.5, on the milk-clotting activity of the T. vulgaris rennet. Ten milliliters of broth concentrated by reverse osmosis was dialysed against 2 liters of the above buffers for 18 hours.

Procedure	Volume (ml)	Milk-clotting Activity (MCU/ml)	Total Activity (MCU)	Protein (mg/ml)	Total Protein (mg)	Specific Activity (MCU/mg protein)
Concentrated broth	10	123.1	1231	10.9	109	11.0
after 18 hours dialysis against:						
Acetate buffer pH 5.6	14	86.4	1209.6	2.6	36.4	33.2
Citrate buffer pH 5.6	14	19.6	274.4	2.4	33.6	8.0
Water pH 5.6	17	72.5	1233	2.4	40.8	30.2
Tris-maleate buffer						
pH 5.6	13	88.9	1155.7	2.8	36.4	31.7
pH 6.0	13	95.3	1239	2.8	36.4	34.1
pH 6.5	14	89.9	1259	2.5	37.8	33.3
pH 7.0	13	97.2	1263	2.7	35.1	36.0
pH 7.5	13	97.0	1261	2.7	35.1	35.9

Table 4 Purification by acetone precipitation of the *T. vulgaris* rennet in broth concentrated by reverse osmosis.

Procedure	Volume (ml)	Milk-clotting Activity (MCU/ml)	Total Activity (MCU)	Protein (mg/ml)	Total Protein (mg)	Specific Activity (MCU/mg protein)
Concentrated broth	30	123.4	3702	9.9	297	12.2
after 18 hours dialysis against 0.02 M acetate buffer, pH 5.6						
Control	43.6	72.9	3193	2.1	91.5	34.9
0-40% acetone	11	8.5	93.5	4.4	48.4	1.9
40-70% acetone	12	152.9	1834	0.65	7.8	233.8

was collected, namely 0 to 0.7. As the enzyme would be dialysed before further purification, it was not dialysed before freeze-drying.

The effects of dialysis on the acetone powder and refreeze-drying are shown in Table 5. There was an approximately three-fold purification by dialysis as previously mentioned but none on refreeze-drying. Further dialysis resulted in a four-fold purification, but there was a substantial loss of activity.

Preliminary investigations indicated that the milk-clotting activity of the microbial enzyme was adversely affected by concentrations of sodium chloride or potassium chloride greater than 0.2 M. Initially a linear gradient elution of 0 to 0.5 M NaCl was used with both resins at pH 6.2 and 7.2. The results were poor. Usually no peak appeared on the absorbance monitor and/or there was very poor recovery (less than 30%)[1] of the enzyme.

Typical results for the prescribed conditions involving DEAE-cellulose resins at pH 6.2 and 7.2 and CM-cellulose resin at pH 7.2 using a linear gradient elution of 0 to 1.0 M NaCl are outlined in Table 6 and in Figures 1, 2 and 3. For comparison, the results are calculated to include the suspension and dialysis of all of the acetone powder, and the chromatography either by DEAE-cellulose or CM-cellulose of aliquots of the dialysed residue.

With both resins, 40 ml of starting buffer was passed through the columns before commencement of elution with sodium chloride. No attempt was made to determine the sodium chloride content in

---

1 The percentages are based on the total activity of the dialysed residue added to the individual columns.

Table 5 Effects of dialysis, freeze-drying and further dialysis on the freeze-dried acetone powder.

Procedure	Volume (ml)	Activity (MCU/ml)	Total Activity (MCU)	Protein (mg/ml)	Total Protein (mg)	Specific Activity (MCU/mg protein)	Yield %	Purification
5 g freeze-dried acetone powder	50	331.2	16560	14.6	730	25.4	100	1.0
after 18 hours dialysis against 0.01 M tris-maleate buffer pH 7.2	55	246.4	13552	3.5	192.5	70.2	82	2.8
after re-freeze drying	15	773.8	11607	11.1	166.5	69.8	71	2.8
after 18 hours dialysis against 0.01 M tris-maleate buffer pH 7.2	22	366.4	6060.8	3.7	74.8	106.7	30	4.2

Table 6 Purification of the T. vulgaris rennet. For the CM-cellulose chromatography, an 8 ml aliquot of the dialysed residue containing 22.4 mg protein and 1378.4 MCU was placed on the column. For the DEAE-cellulose chromatography, an 10 ml aliquot containing 28 mg protein and 1723 MCU was used. The protein and activity recovered are from these aliquots.

Procedure	Volume (ml)	Activity (MCU/ml)	Total Activity (MCU)	Protein (mg/ml)	Total Protein (mg)	Specific Activity (MCU/mg protein)	Yield %	Purification	Milk-Clotting / Proteolytic Activity (MCU/ug tyrosine)
crude extract	28175	26.3	741002	3.4	95795	7.7	100	1.0	
concentration by reverse osmosis	7615	70.6	537454	6.3	47834	11.2	72	1.4	
90 g freeze-dried acetone powder	1930	210.0	405300	7.9	15247	26.6	55	3.4	
after 18 hours dialysis against 0.01 M tris-maleate buffer, pH 7.2	2120	172.3	365276	2.8	5936	61.5	49	7.9	1.20
CM-cellulose chromatography pH 7.2	32	20.8	665.6	0.024	0.768	866.6	24	112.5	0.27
DEAE-cellulose chromatography pH 7.2	60	25.4	1524	0.181	10.8	140.3	42	18.2	0.95
DEAE-cellulose chromatography pH 6.2	55	26.8	1474	0.185	10.2	144.5	38	18.7	1.10

Figure 1 Chromatographic pattern of the T. vulgaris rennet on CM-cellulose (2.5 x 19 cm). (—o—o—) Milk-clotting activity, (—■—■—) % $T_{280\ nm}$, sodium chloride concentration - solid line. Elution buffer, 0.01 M tris-maleate buffer (pH 7.2).

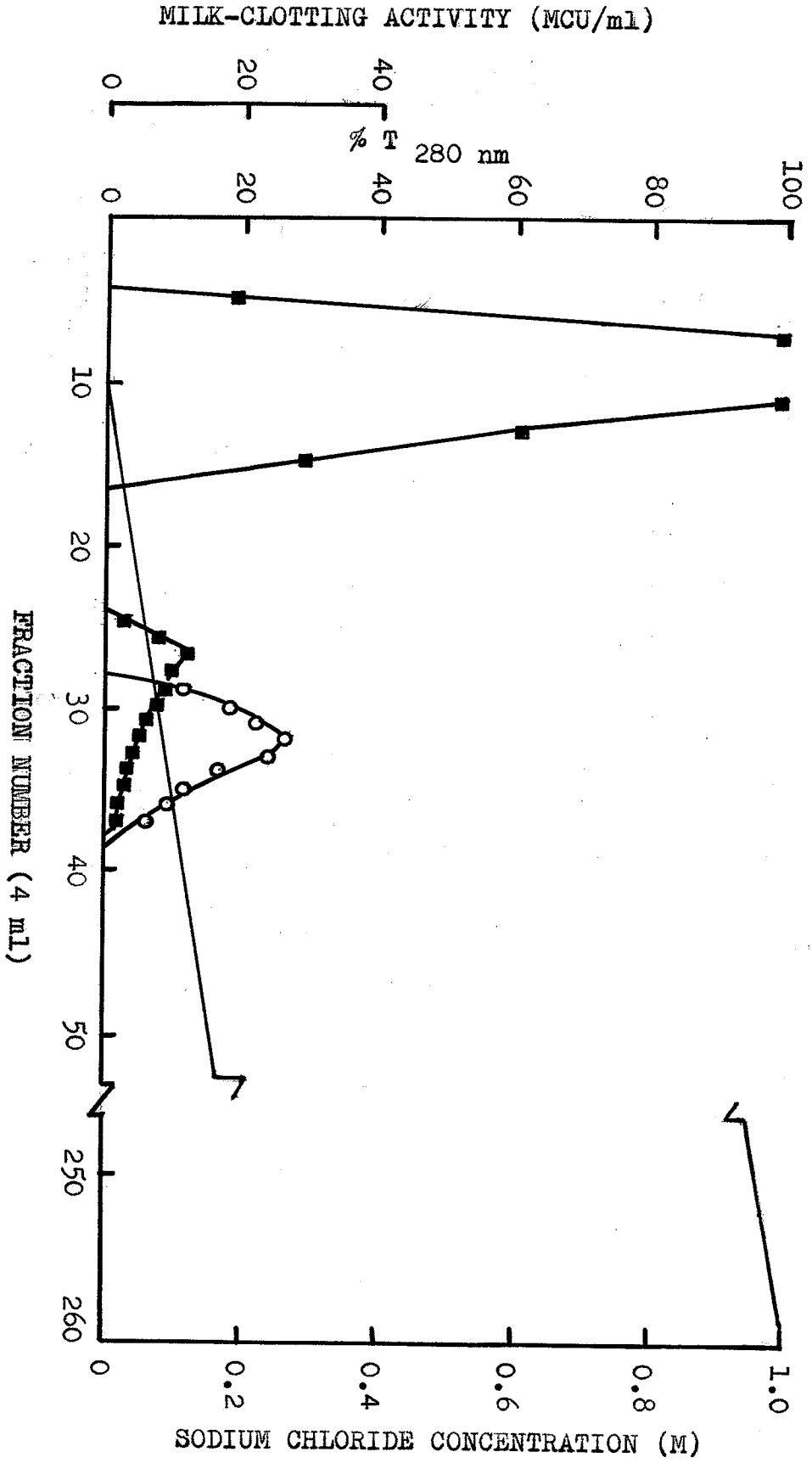

MILK-CLOTTING ACTIVITY (MCU/ml)
0
20
40
% T 280 nm
0
20
40
60
80
100
10
20
30
40
50
250
260
FRACTION NUMBER (4 ml)
0
0.2
0.4
0.6
0.8
1.0
SODIUM CHLORIDE CONCENTRATION (M)

Figure 2 Chromatographic pattern of the T. vulgaris rennet on DEAE-cellulose (2.5 x 19 cm). (—o—o—) Milk-clotting activity, (—■—■—) % $T_{280\ nm}$, sodium chloride concentration - solid line. Elution buffer, 0.01 M tris-maleate buffer (pH 7.2).

MILK-CLOTTING ACTIVITY (MCU/ml)
0
20
40
60
% T 280 nm
0
20
40
60
80
100
10
20
30
40
50
200
210
FRACTION NUMBER (5 ml)
0
0.2
0.4
0.6
0.8
1.0
SODIUM CHLORIDE CONCENTRATION (M)

Figure 3 Chromatographic pattern of the T. vulgaris rennet on DEAE-cellulose (2.5 x 19 cm). (—o—o—) Milk-clotting activity, (—■—■—) % $T_{280\ nm}$, sodium chloride concentration - solid line. Elution buffer, 0.01 M tris-maleate buffer (pH 6.2).

MILK-CLOTTING ACTIVITY (MCU/ml)
0
20
40
60
% T 280 nm
0
20
40
60
80
100
10
20
30
40
50
200
210
FRACTION NUMBER (5 ml)
0
0.2
0.4
0.6
0.8
1.0
SODIUM CHLORIDE CONCENTRATION (M)

each fraction. Therefore, the gradient illustrated on the chromatographic pattern in Figures 1, 2 and 3 is theoretical.

There was very little difference in the results with DEAE-cellulose resins at pH 6.2 and 7.2. The pigment remained on the column even after successive washings with 1 M NaCl. The enzyme recovery was 80 to 95%[1] and there was no significant change in the ratio of milk-clotting activity to proteolytic activity. The fold-purification, however, was from 2.2 to 2.5 over the dialysis residue.

There was no recovery of the enzyme at pH 6.2 with CM-cellulose. However, trials with the resin at pH 7.2 were successful. Purification ranged from 11- to 14.7- fold over the dialysis residue. Pigmented matter was recovered in the first peak. Unfortunately milk-clotting activity recovery was low (42 to 52%[1]) and the milk-clotting / proteolytic activity ratio decreased approximately 5-fold.

The purification by the methods described is outlined in Table 6. With CM-cellulose there was an approximately 112-fold purification over the crude extract, whilw with DEAE-cellulose there was a 18-fold purification. The chromatographically-active fraction from both resins was colorless and odorless compared to the pigmented unpleasant-smelling acetone powder.

Further purification attempts by gel filtration on Sephadex G100 and G150 using 0.01 M tris-maleate buffer, pH 7.2, were unsuccessful.

Polyacrylamide Gel Electrophoresis

The location of protein bands and proteolytic activity following electrophoresis in a 7.7% polyacrylamide gel at pH 6.2 is shown

in Figure 4. Protein bands were located by staining with 0.02% Coomassie Brilliant Blue while the proteolytically active bands were determined by staining with 1% amido black. For comparison, the electrophoretic results are illustrated by line drawings with the R_f values adjusted to a standard absolute mobility. R_f values for each band were reproducible to within 7%. The heterogeneity of both proteins is evident.

Commercial calf rennet exhibited three proteolytic bands (R_f values 0.305, 0.690 and 0.762) but only two protein bands. However, the width of the bands and their location suggest that one protein band contains two proteolytically-active components.

For the crude microbial rennet, there were three distinct proteolytic bands (R_f values 0.138, 0.374 and 0.496). The closeness of the protein bands and the width of the proteolytic bands made it difficult to identify which protein bands had activity, espeially for the slow-moving components.

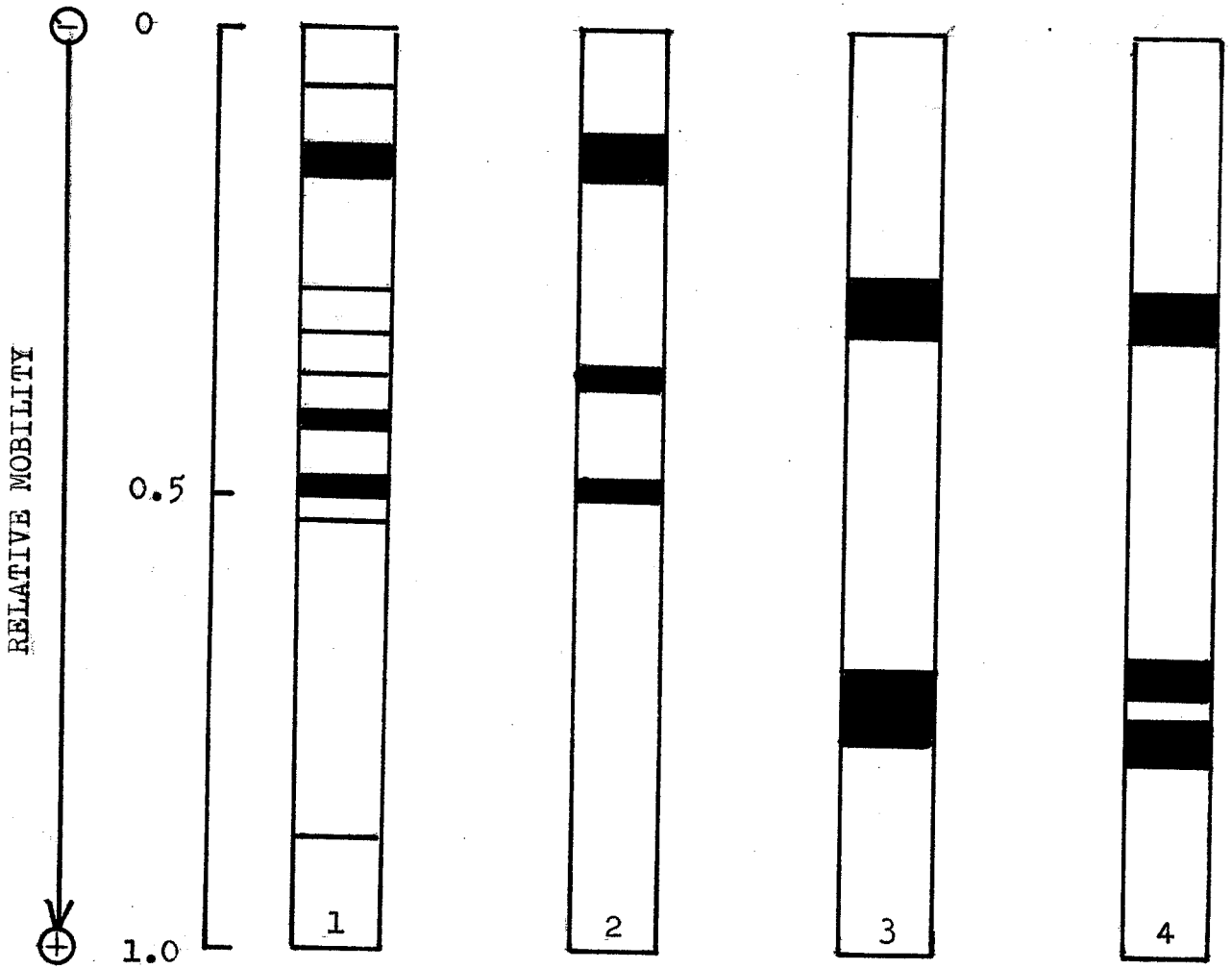
Only one of the proteolytic microbial bands possessed a similar R_f value to an active proteolytic calf rennet band.

Electrophoresis of the fractions obtained from DEAE- and CM-cellulose chromatography was unsuccessful due to insufficient protein and/or activity.

Proteolytic Activity

Variations in proteolytic activity due to pH for commercial calf rennet and dialysis residue of the microbial rennet are illustrated in Figure 5. Proteolytic action on casein was measured only above pH 5.5 as activity on casein in the range 5.5 to 7.5 is more relevant in the cheese process.

Figure 4 Line drawings of isoenzymes and proteolytically - active zones of T. vulgaris rennet and of commercial calf rennet.



T. vulgaris Rennet

1. Protein Bands
2. Proteolytically-Active Zones

Commercial Calf Rennet

3. Protein Bands
4. Proteolytically-Active Zones

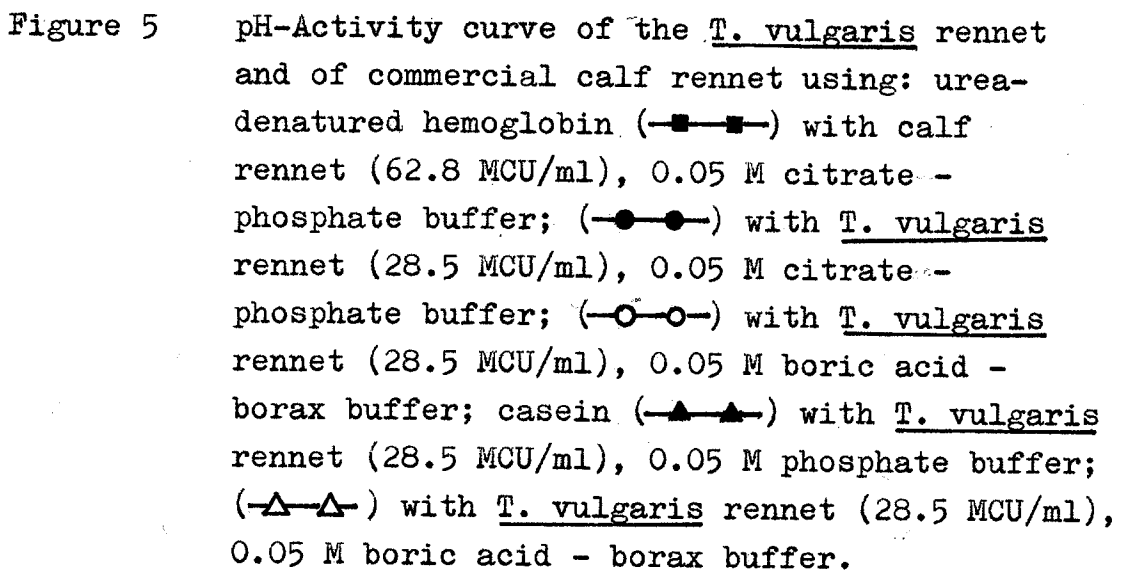
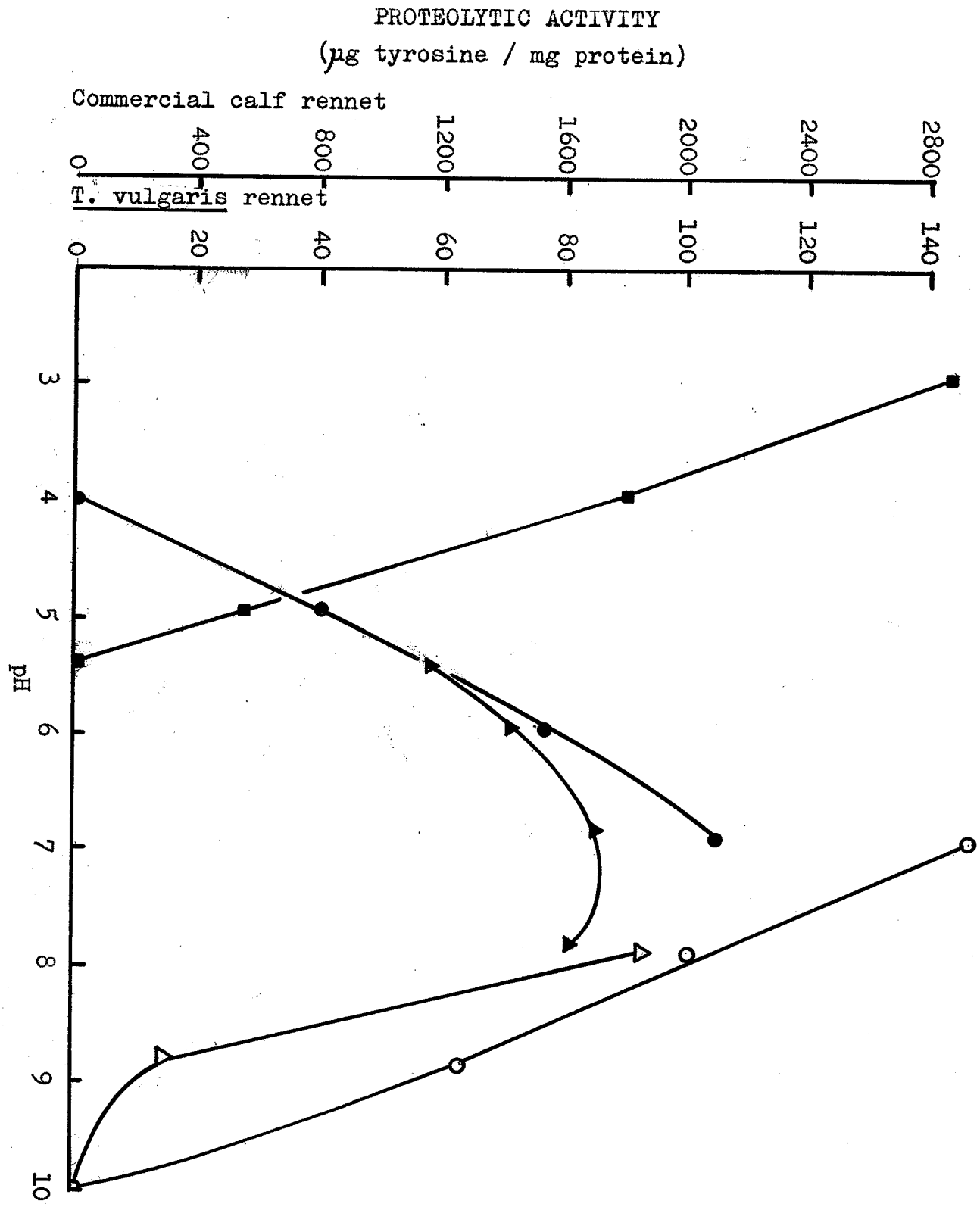


Figure 5 pH-Activity curve of the T. vulgaris rennet and of commercial calf rennet using: urea-denatured hemoglobin (—■—■—) with calf rennet (62.8 MCU/ml), 0.05 M citrate - phosphate buffer; (—●—●—) with T. vulgaris rennet (28.5 MCU/ml), 0.05 M citrate - phosphate buffer; (—○—○—) with T. vulgaris rennet (28.5 MCU/ml), 0.05 M boric acid - borax buffer; casein (—▲—▲—) with T. vulgaris rennet (28.5 MCU/ml), 0.05 M phosphate buffer; (—△—△—) with T. vulgaris rennet (28.5 MCU/ml), 0.05 M boric acid - borax buffer.


PROTEOLYTIC ACTIVITY
(µg tyrosine / mg protein)
Commercial calf rennet
0
400
800
1200
1600
2000
2400
2800
T. vulgaris rennet
0
20
40
60
80
100
120
140
pH
3
4
5
6
7
8
9
10

Calf rennet, both concentrated and diluted, exhibited no activity above pH 6 with either 1% urea-denatured hemoglobin or 1% casein. The greatest activity was demonstrated at pH 3 with hemoglobin as the substrate.

The microbial rennet, however, was only active between pH 5 and 9. There was a relatively sharp optimum (pH 7) with hemoglobin but a broader optimum (pH 7 to 8) with casein.

The buffering system used also had a definite effect on activity. The activities in the presence of borate buffer tended to be higher than those in the presence of citrate-phosphate buffer or phosphate buffer.

Effect of Substrates

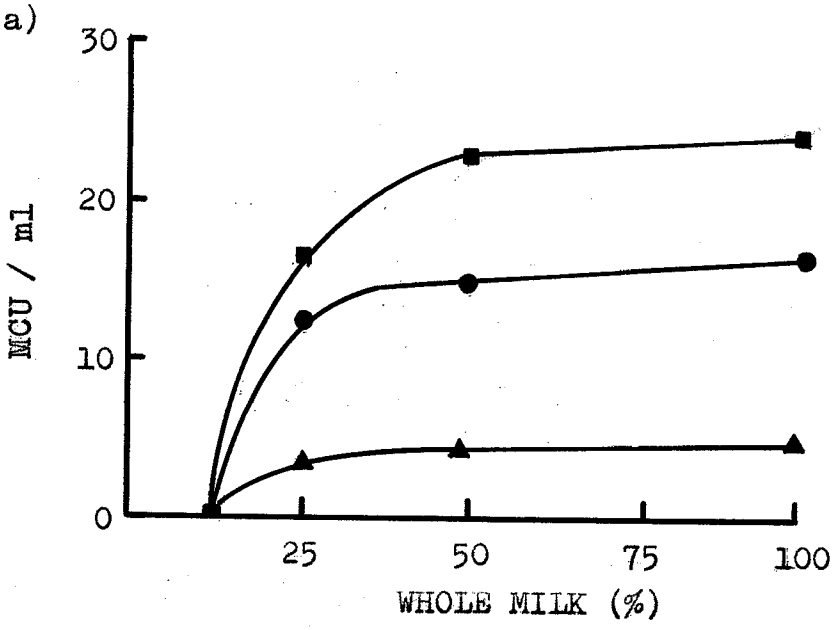
The effect of diluting whole milk with or without calcium chloride at different concentrations of microbial rennet is shown in Figure 6 a and b. With the exception that activity was higher in the presence of calcium chloride, there was no noticeable difference in the pattern of the two substrates on activity.

Below 12.5% milk, precipitation was visible, but it was insufficient to stop the timer. With 25% milk, there was a delay (approximately 10 seconds) between visible precipitation and clotting as measured by the instrument. A maximum difference of 3 seconds was noted among replicate determinations at 50% milk and at 100% milk. Also between 50% milk and 100% milk, very little difference in activity (MCU/ml) was noted.

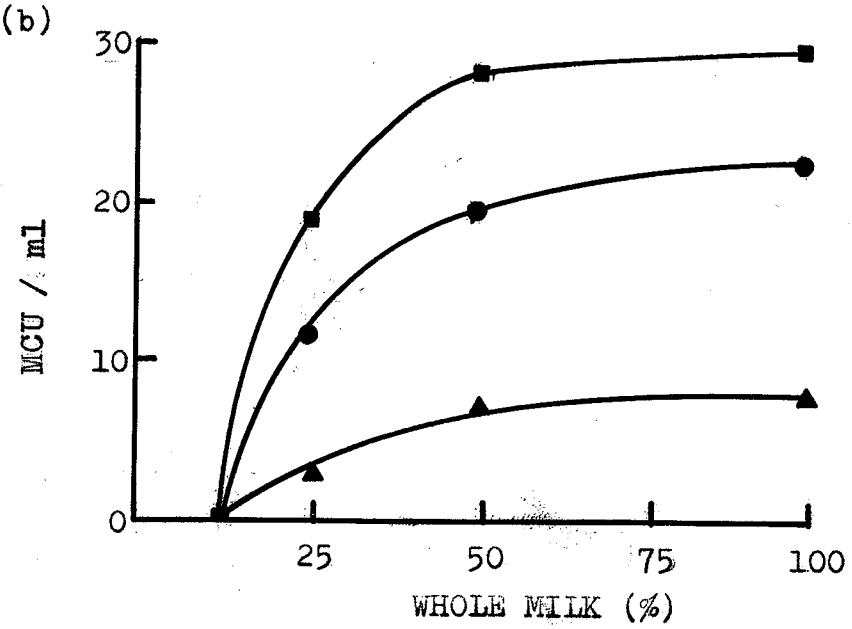
Next, three different substrates were examined - 100% milk, pH 5.6; 50% milk-acetate buffer, pH 5.6; and 50% milk-tris-maleate buffer, pH 5.6. The concentration of calcium chloride was 0.01 M in all three.

Figure 6 Effect of diluting whole milk without (a) or with (b) 0.01 M $CaCl_2$ on milk-clotting activity at different concentrations of T. vulgaris rennet expressed as mg protein / ml - (-■-■-) 12.53 mg protein / ml, (-●-●-) 1.26 mg protein / ml, (-▲-▲-) 0.31 mg protein / ml.

(a)


30
20
10
0
MCU / ml
25
50
75
100
WHOLE MILK (%)

(b)


30
20
10
0
MCU / ml
25
50
75
100
WHOLE MILK (%)

For the three microbial rennet preparations (Figure 7 a and b) there were no major differences in activity among the three substrates. However, this was not true for the commercial calf rennet (Figure 7 c). Clotting was generally slower with the 50% milk-tris-maleate buffer substrate, especially at higher enzyme concentrations.

Effect of Enzyme Concentration

The effects of enzyme concentration (µg protein/ml) on milk-clotting activity for the microbial rennet (acetone powder and dialysis residue) and for commercial calf rennet are also illustrated in Figures 7 a, b and c.

As the enzyme concentration is increased, there is a progressive increase in activity. However the relative rate of increase decreases as the enzyme concentration reaches a limiting amount - 2.5 mg protein / ml for the acetone microbial powder, 500 µg protein / ml for the dialysis residue, and 61 µg protein / ml for commercial calf rennet.

The results also indicate that the limits in which the milk-clotting activity is proportional to enzyme concentration as well as the clotting strength (MCU/mg protein) depends upon the purity and the source of the enzyme (Table 7). There was insufficient data to determine the range for the DEAE and CM fractions. The clotting strength reported for these fractions is based on the chromatographic results (Table 6). For the microbial rennet to have the same clotting strength as commercial calf rennet, approximately 33 times as much dialysis residue is necessary. After chromatography by either DEAE-cellulose or CM-cellulose approximately 14 times or 2.3 times as much, respectively, is required.

Figure 7a Effect of various concentrations of the T. vulgaris rennet, acetone powder, on milk-clotting activity with different substrates - (—O—O—) Substrate 1, (—□—□—) Substrate 2, (—△—△—) Substrate 3.

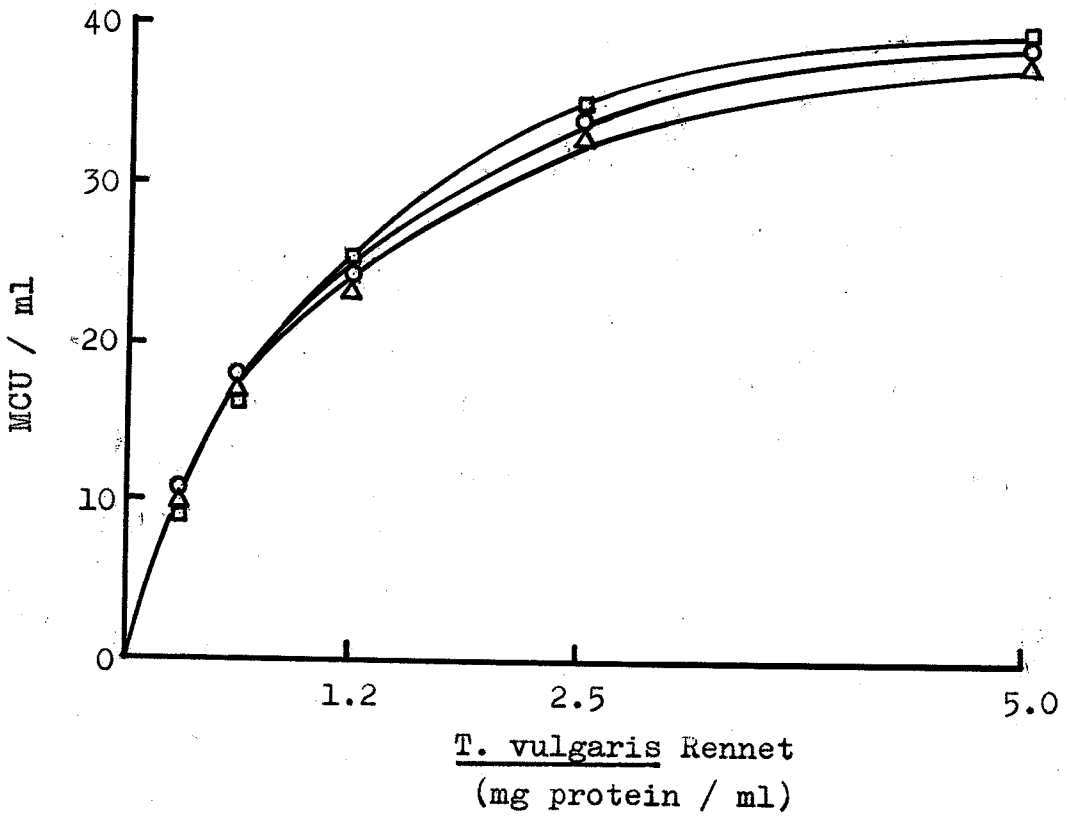

40
30
20
10
0
MCU / ml
1.2
2.5
5.0
T. vulgaris Rennet
(mg protein / ml)

Figure 7b Effect of various concentrations of the T. vulgaris rennet, dialysis residue and DEAE fraction, on milk-clotting activity with different substrates - (—o—o—) Substrate 1, (—□—□—) Substrate 2, (—△—△—) Substrate 3.

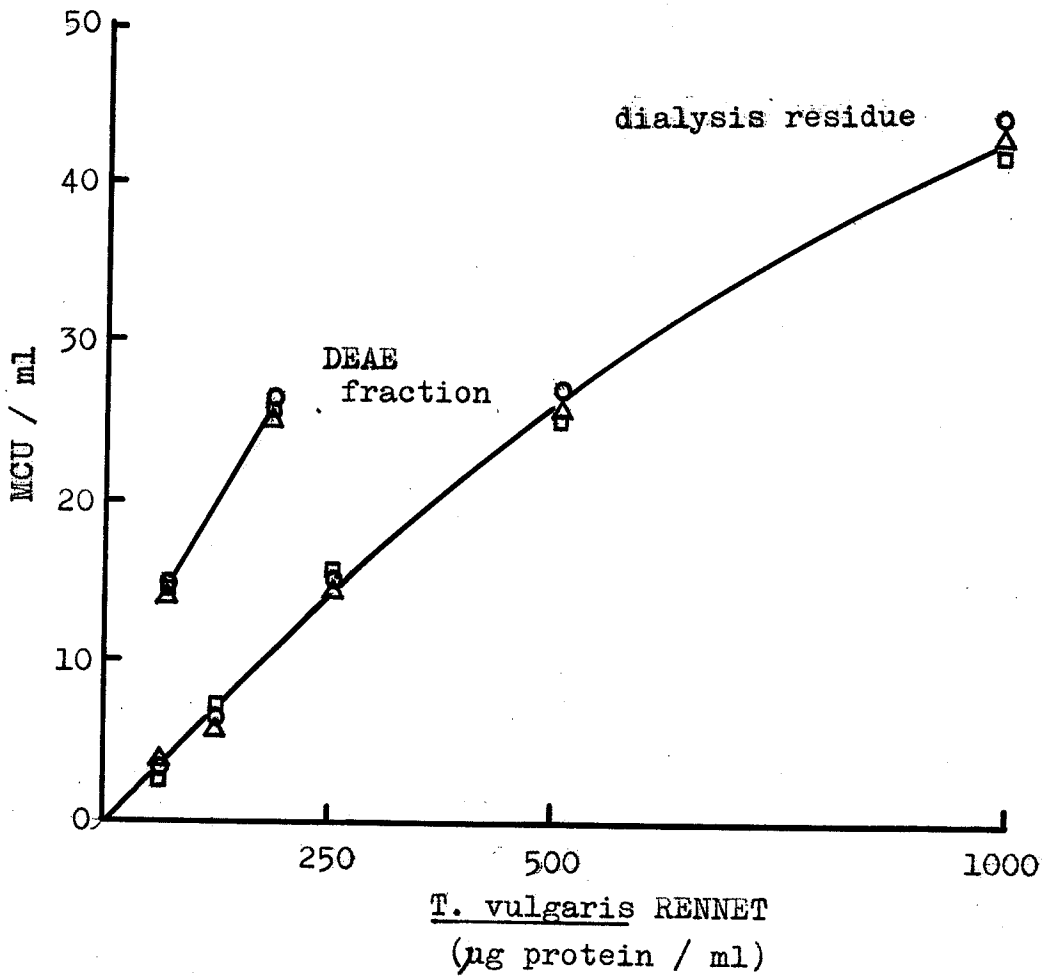

50
40
30
20
10
0
MCU / ml
dialysis residue
DEAE
fraction
250
500
1000
T. vulgaris RENNET
(µg protein / ml)

Figure 7c Effect of various concentrations of commercial calf rennet on milk-clotting activity with different substrates - (—○—○—) Substrate 1, (—□—□—) Substrate 2, (—△—△—) Substrate 3.

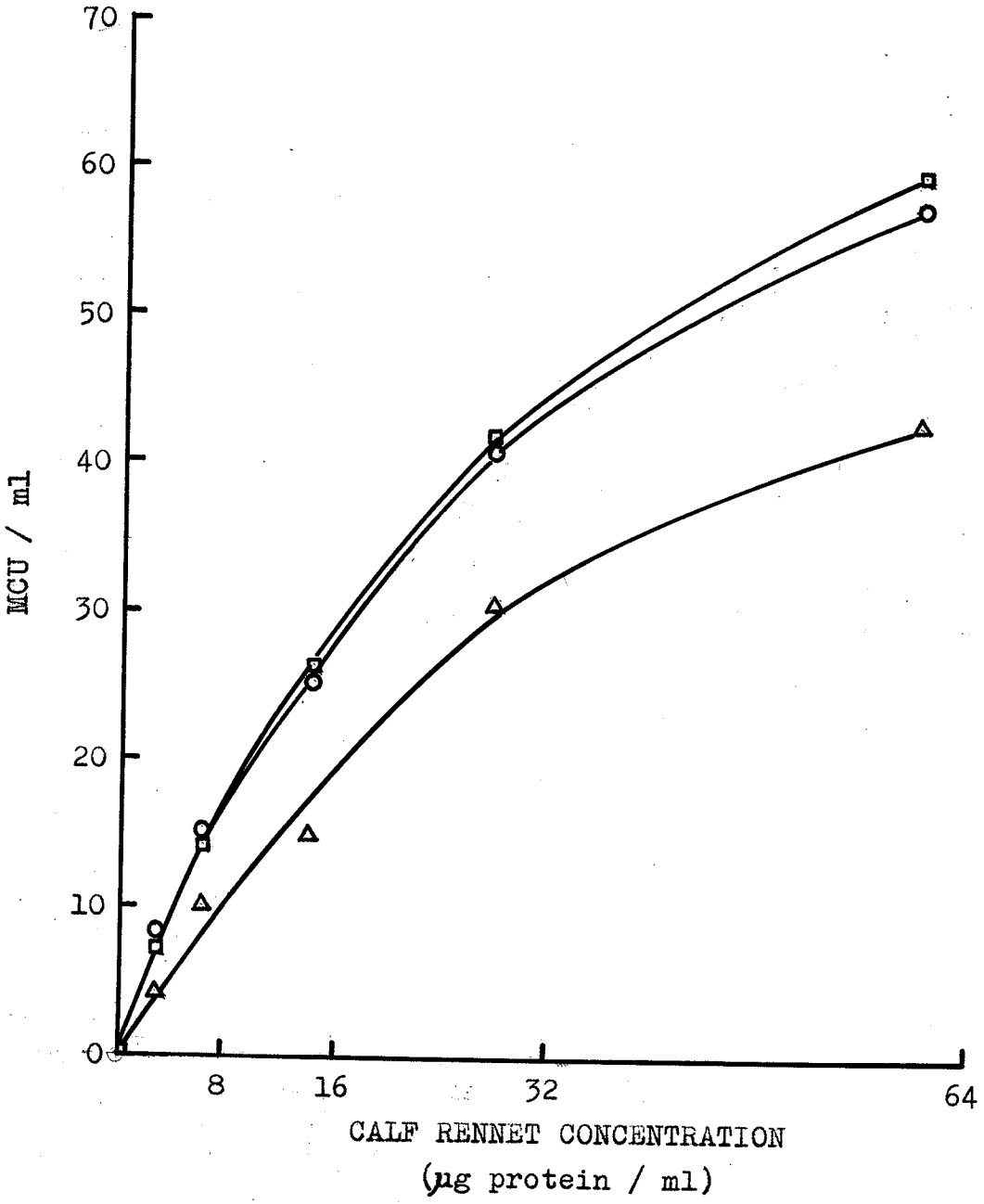

70
60
50
40
30
20
10
0
MCU / ml
8
16
32
64
CALF RENNET CONCENTRATION
(µg protein / ml)

Table 7 Enzyme concentrations (expressed as µg protein / ml) at which milk-clotting activity is proportional to the enzyme concentration and the clotting strength (expressed as MCU / mg protein) for different preparations of T. vulgaris rennet and for commercial calf rennet.

Rennet	Limits of Proportionality (µg enzyme protein / ml)	Clotting Strength (MCU / mg enzyme protein)
T. vulgaris rennet		
acetone powder	300 - 1200	27.4
dialysis residue	62 - 250	60.0
DEAE fraction		140.4
CM fraction		866.6
Calf rennet		
commercial	4 - 30	1974.0

pH Profile

Varying the pH of the milk had a definite effect on the clotting time for different concentrations of microbial rennet and commercial calf rennet (Figures 8 a, b and c).

Both had optimum activity in the region of 5.6 but the decline in activity at higher pH values was sharper for the commercial calf rennet than for the microbial rennet. Little or no activity was exhibited by either enzyme above pH 7.

It was difficult to determine clotting activity below pH 5.5 as slight precipitation occurred on mixing the milk and buffer. At pH 5 there was instantaneous clotting without the addition of enzyme.

The range of activity, as expected, was dependent upon enzyme concentration.

pH Stability

The sensitivity of the microbial rennet and commercial calf rennet to pH is illustrated in Figure 9.

Calf rennet was stable between pH 3 and 7 while the microbial rennet was stable between pH 5.6 and 11. It was, however, as susceptible to acidic denaturation as calf rennet was to alkaline denaturation. The milk-clotting DEAE fraction, although stable, was more sensitive to pH than the cruder form. The CM fraction followed the same pattern as the DEAE fraction.

As no major discrepancies were found between results obtained using substrates 1 and 2, the only results illustrated are those obtained with substrate 1.

Figure 8a Effect of varying the pH of milk on milk-clotting activity at different concentrations of the dialysis residue of the T. vulgaris rennet.
(—□—□—) 500 µg protein / ml
(—△—△—) 250 µg protein / ml
(—○—○—) 125 µg protein / ml

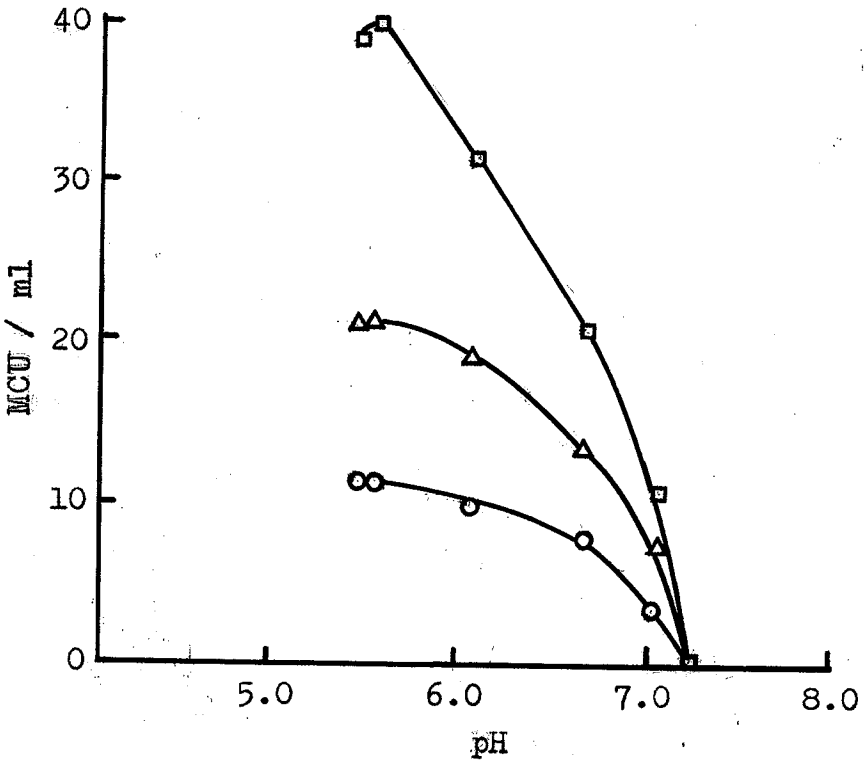

40
30
20
10
0
MCU / ml
5.0
6.0
7.0
8.0
pH

Figure 8b Effect of varying the pH of milk on milk-clotting activity at different concentrations of the DEAE fraction of the T. vulgaris rennet.
(—□—□—) 73.0 µg protein / ml
(—○—○—) 36.5 µg protein / ml

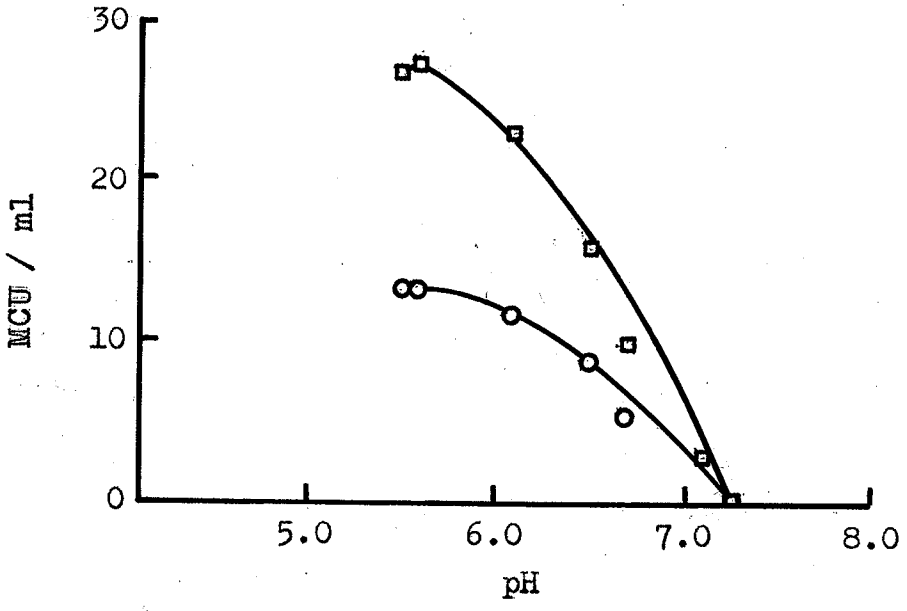

30
20
10
0
MCU / ml
5.0
6.0
7.0
8.0
pH

Figure 8c Effect of varying the pH of milk on milk-clotting activity at different concentrations of commercial calf rennet.
(—□—□—) 122.5 µg protein / ml
(—△—△—) 30.6 µg protein / ml
(—○—○—) 7.6 µg protein / ml

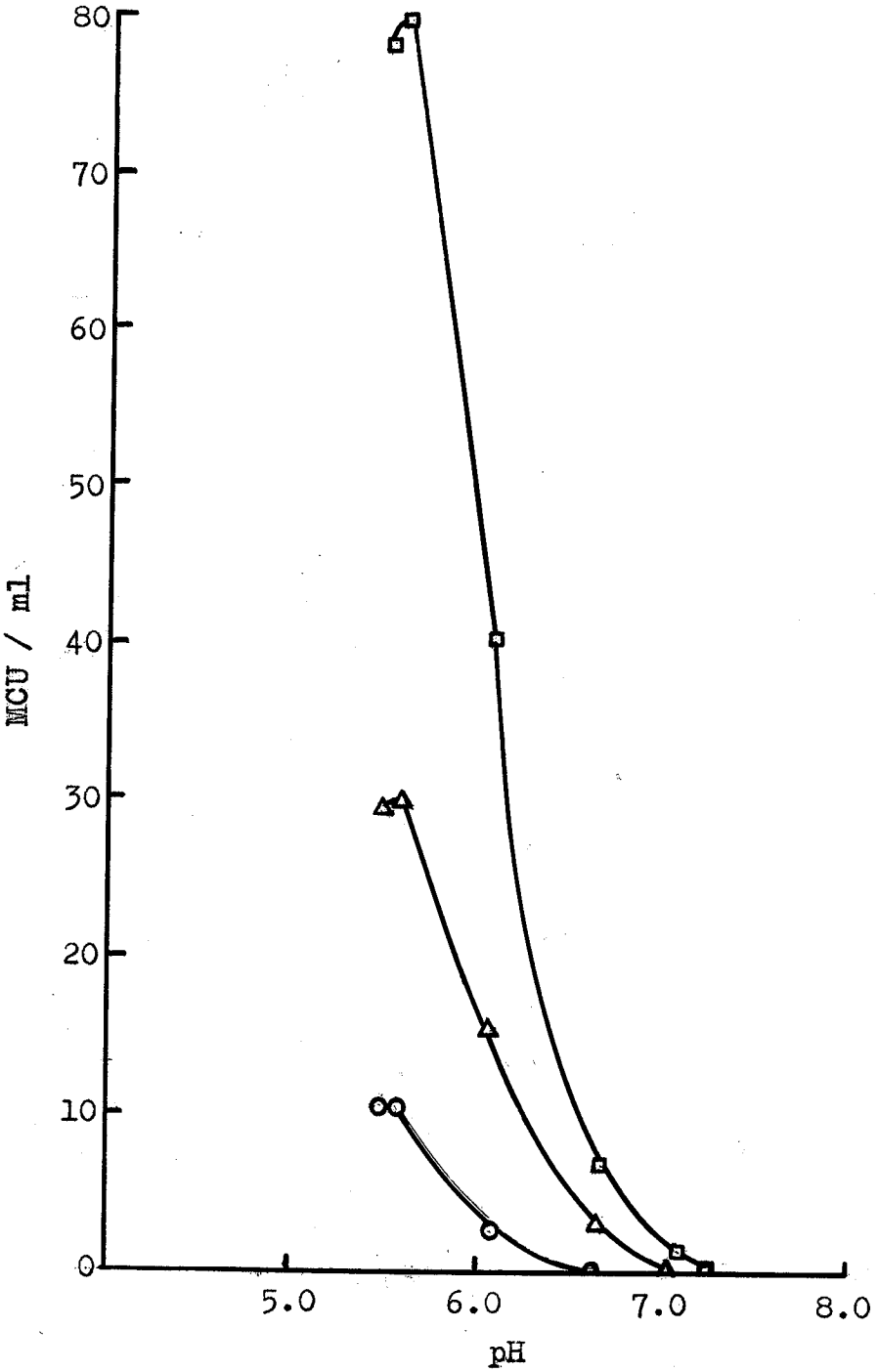

80
70
60
50
40
30
20
10
0
MCU / ml
5.0
6.0
7.0
8.0
pH

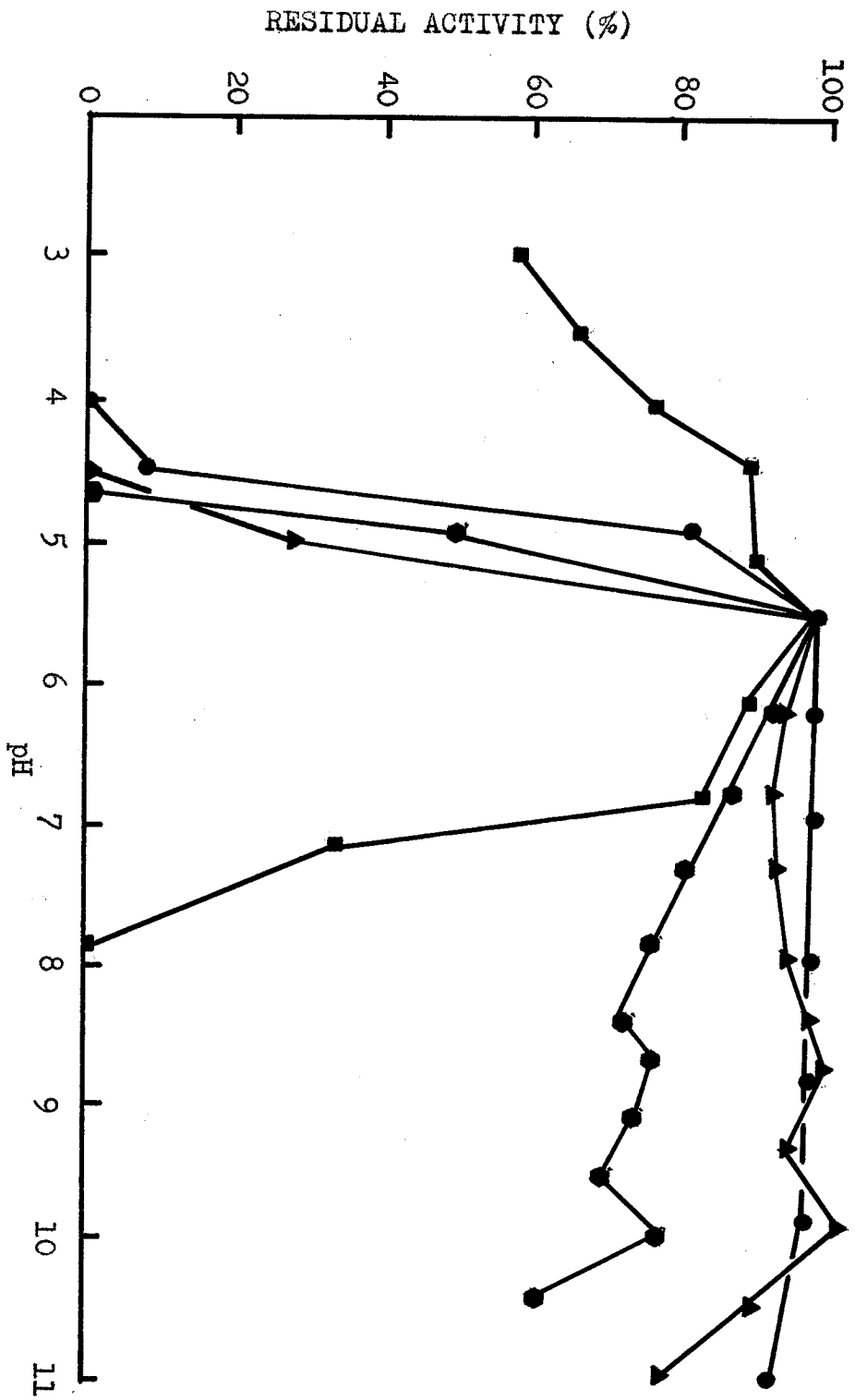
Figure 9 Enzyme stability on exposure to pH at room temperature.

(-●—●-) T. vulgaris rennet - 1 hour exposure (dialysis residue)

(-▲—▲-) T. vulgaris rennet - 2 hour exposure (dialysis residue)

(-●—●-) T. vulgaris rennet - 2 hour exposure (DEAE fraction)

(-■—■-) Commercial calf rennet - 2 hour exposure


RESIDUAL ACTIVITY (%)
0
20
40
60
80
100
pH
3
4
5
6
7
8
9
10
11

Heat Stability

The recovery of enzymatic activity after exposure to different temperatures for specific time periods is outlined in Table 8. The microbial rennet was stable after sixty minutes' exposure to temperatures as high as 55°C. Higher temperatures (60°C, 65°C) were detrimental.

Results, as for pH stability, were independent of the substrate used.

Storage Stability

The data obtained on the storage stability of the dialysed residue indicated it to be more stable at -10°C and at 10°C than at 25°C (Figure 10). At 25°C, the milk-clotting activity rapidly decreased over the 24-day period.

The acetone powder stored at 10°C exhibited no loss of activity after one year. Solutions of the DEAE and CM fractions were stable for two weeks at 10°C.

Effect of Metal Ions and of Cyanide

The general pattern, activation at low concentration followed by inhibition at higher concentrations, was obtained after incubating the microbial rennet and commercial calf rennet for 10 minutes at 37°C with different concentrations of NaCl, KCl, $CaCl_2$ and $BaCl_2$ (Figures 11, 12, 13 and 14).

An important observation was the significant difference in clotting time between substrates 1A and 2A for calf rennet but not for the microbial rennet. The activities of substrates 1 and 2 for either enzyme were not significantly different.

Table 8 Heat stability of the T. vulgaris rennet (dialysed residue).

Temperature (°C)	Time (minutes)	% Residual Activity Substrate 1	Substrate 2
37		100 %	100 %
45	30	100	100
	60	98.1	97.5
	120	84.4	86.5
50	15	81.7	82.6
	30	76.4	74.7
	60	55.8	57.4
55	15	58.6	57.9
	30	42.3	44.4
	60	17.3	18.5
60	10	44.7	46.9
	20	16.4	18.9
	40	0	0
65	5	0	0

Figure 10 Storage stability of the T. vulgaris rennet (dialysis residue)
(—■—■—) 25°C
(—▲—▲—) 10°C
(—●—●—) -10°C

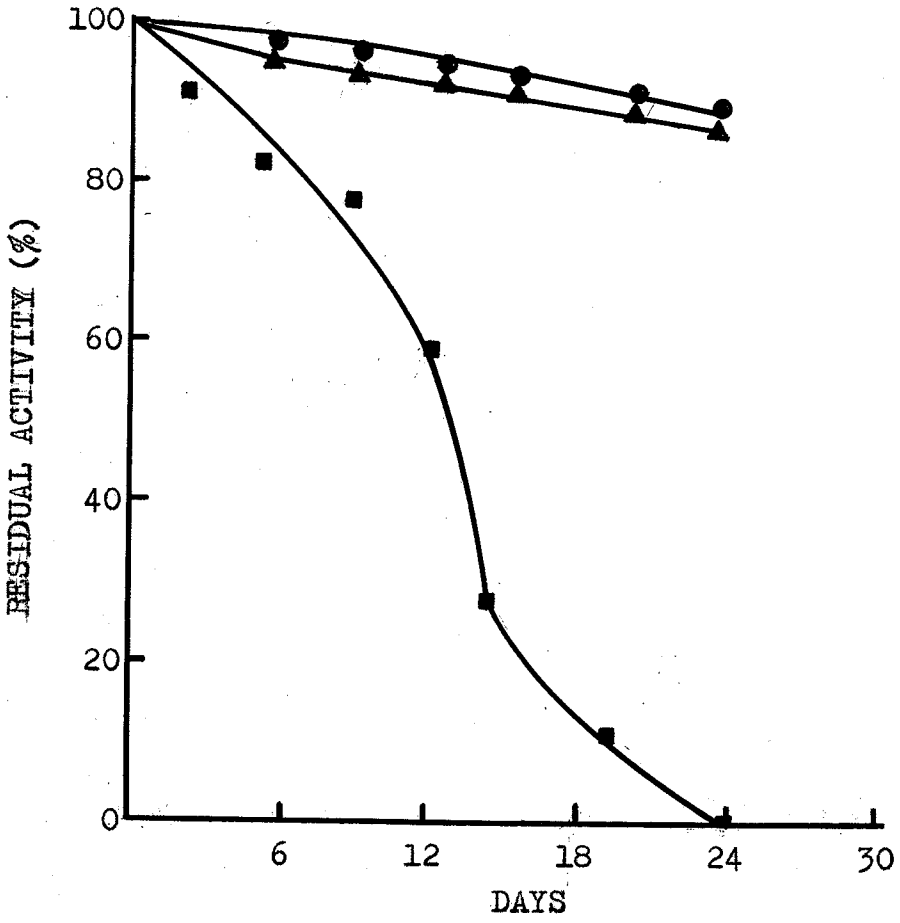

100
80
60
40
20
0
RESIDUAL ACTIVITY (%)
6
12
18
24
30
DAYS

Figure 11 Effect of different concentrations of sodium chloride on milk-clotting activity of (—●—●—) T. vulgaris rennet and of (—■—■—) commercial calf rennet with different substrates - (a) Substrate 2A, (b) Substrate 2, (c) Substrate 1A, (d) Substrate 1.

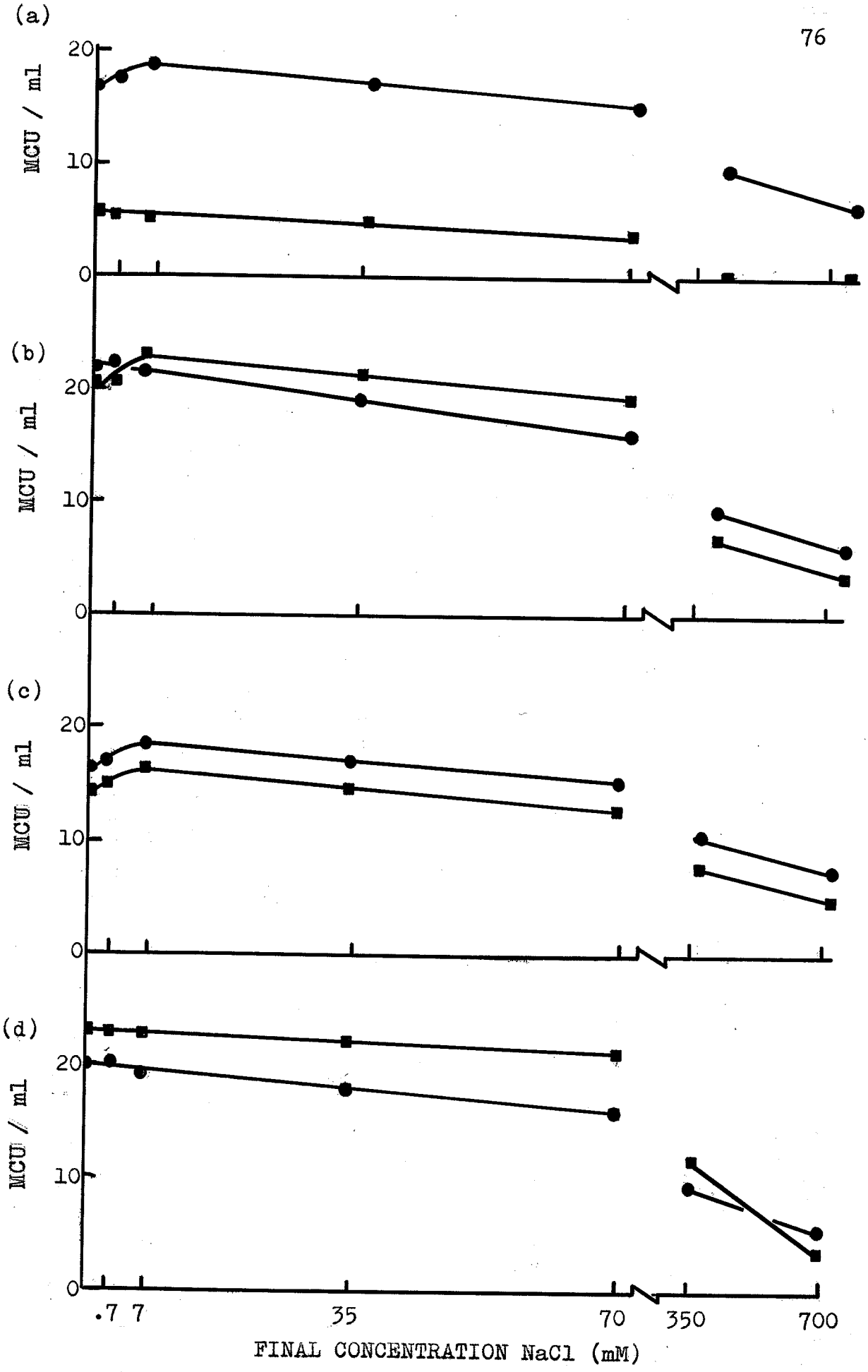

(a)
(b)
(c)
(d)
MCU / ml
20
10
0
.7
7
35
70
350
700
FINAL CONCENTRATION NaCl (mM)

Figure 12 Effect of different concentrations of potassium chloride on milk-clotting activity of (—●—●—) T. vulgaris rennet and of (—■—■—) commercial calf rennet with different substrates - (a) Substrate 2A, (b) Substrate 2, (c) Substrate 1A, (d) Substrate 1.

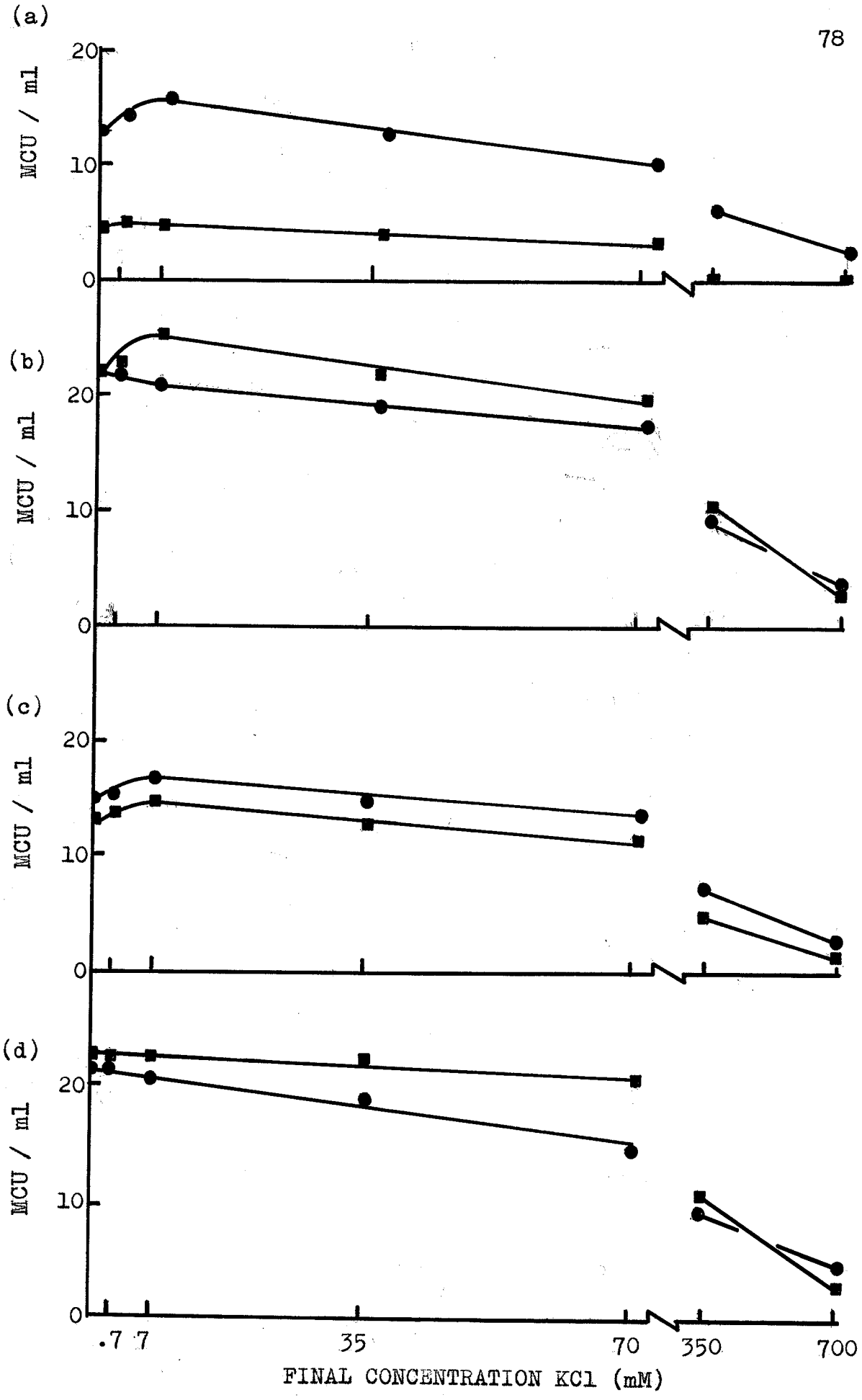
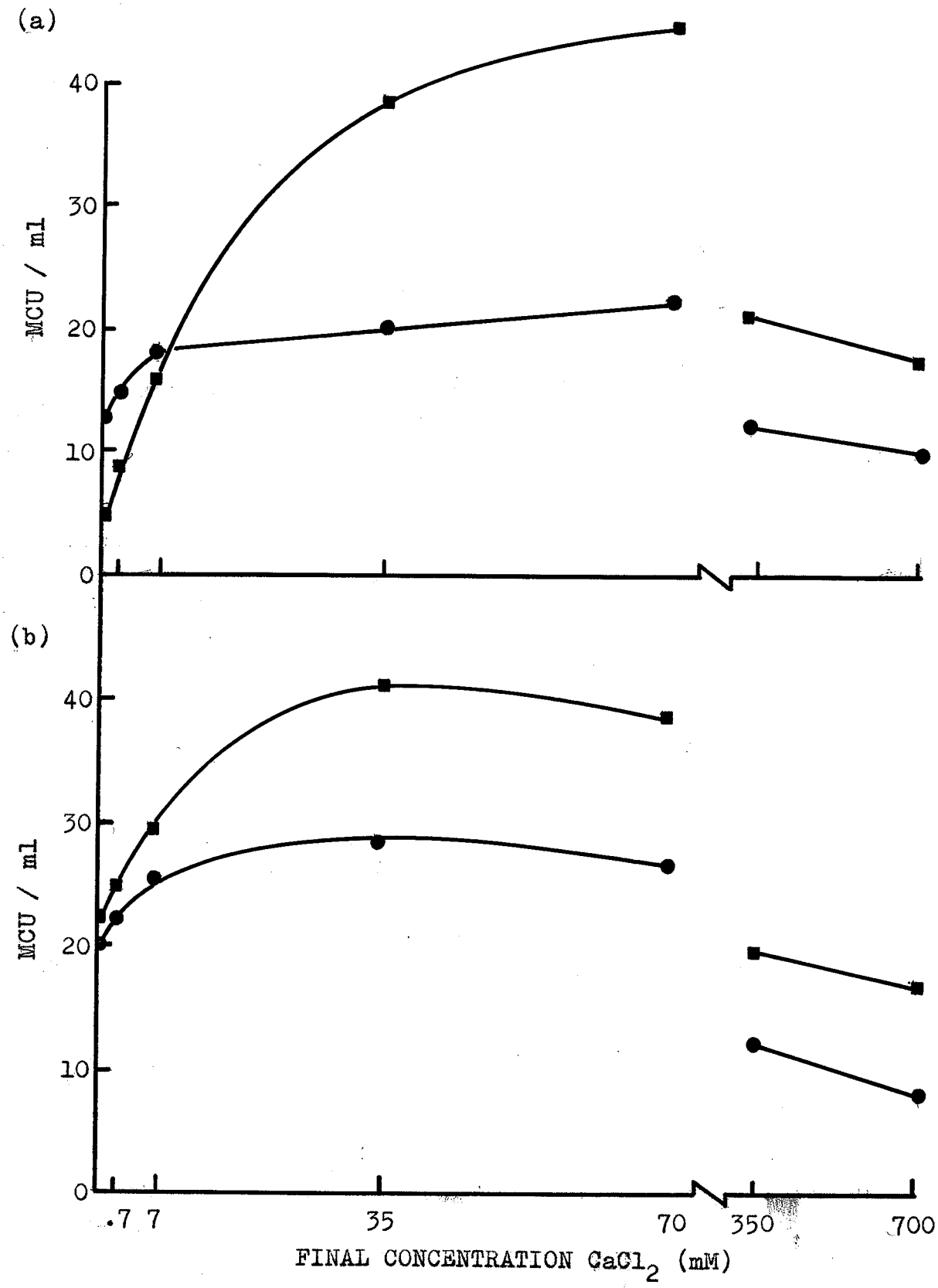

(a)
(b)
(c)
(d)
MCU / ml
20
10
0
.7
7
35
70
350
700
FINAL CONCENTRATION KCl (mM)

Figure 13 Effect of different concentrations of calcium chloride on milk-clotting activity of (—●—●—) T. vulgaris rennet and of (—■—■—) commercial calf rennet with different substrates - (a) Substrate 2A, (b) Substrate 2, (c) Substrate 1A, (d) Substrate 1.


(a)
40
30
20
10
0
MCU / ml
(b)
40
30
20
10
0
MCU / ml
.7
7
35
70
350
700
FINAL CONCENTRATION $CaCl_2$ (mM)

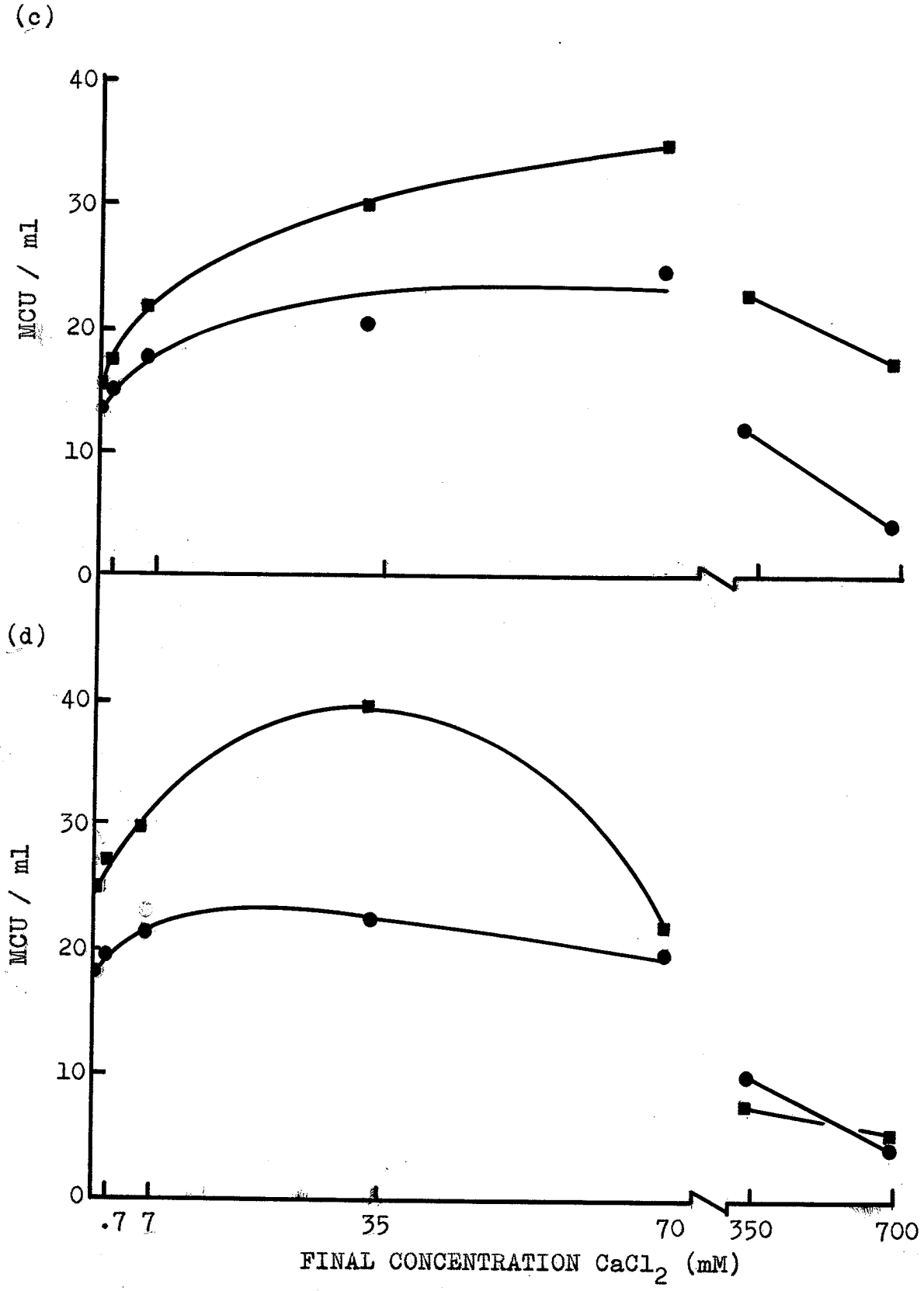
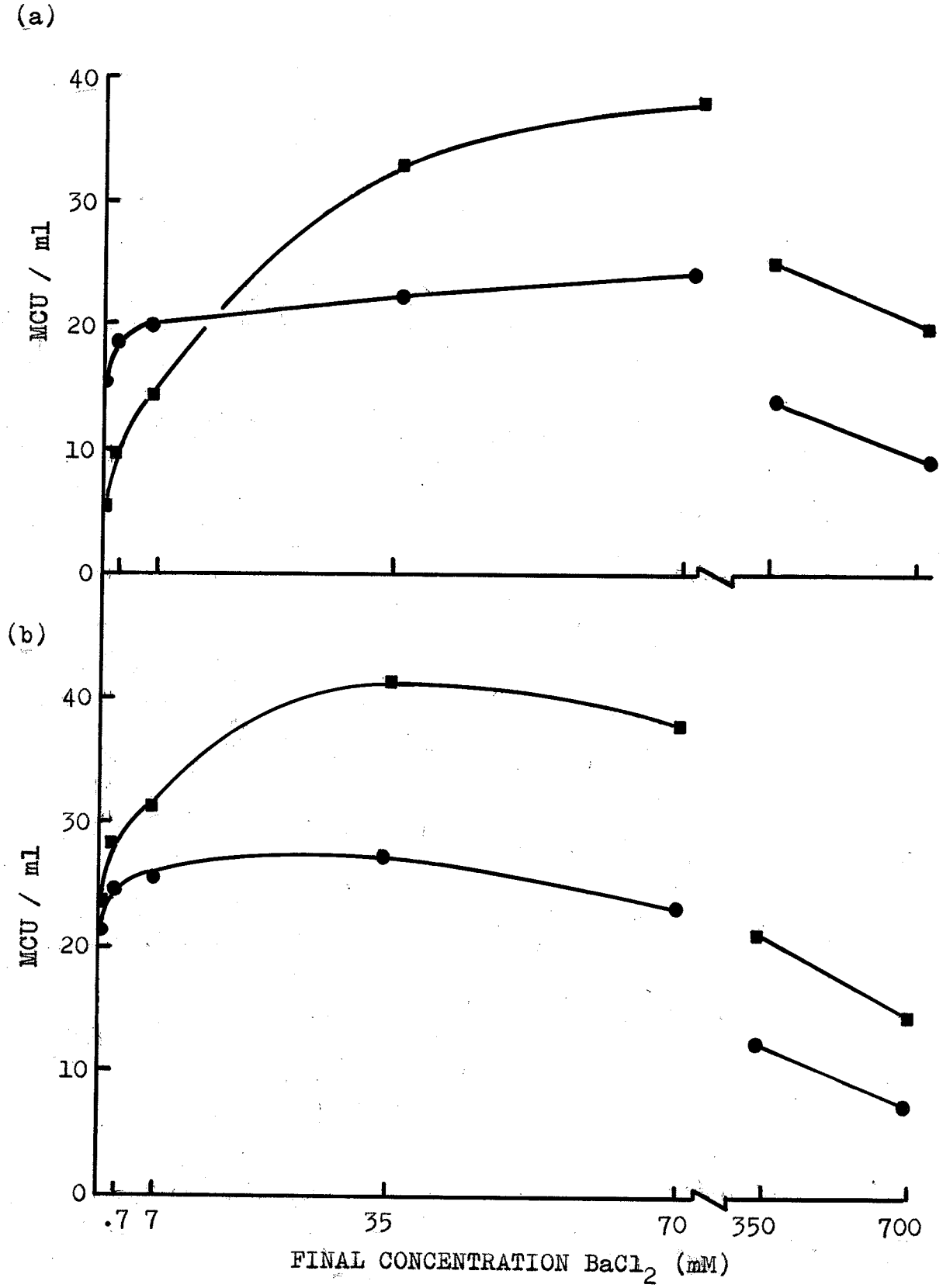
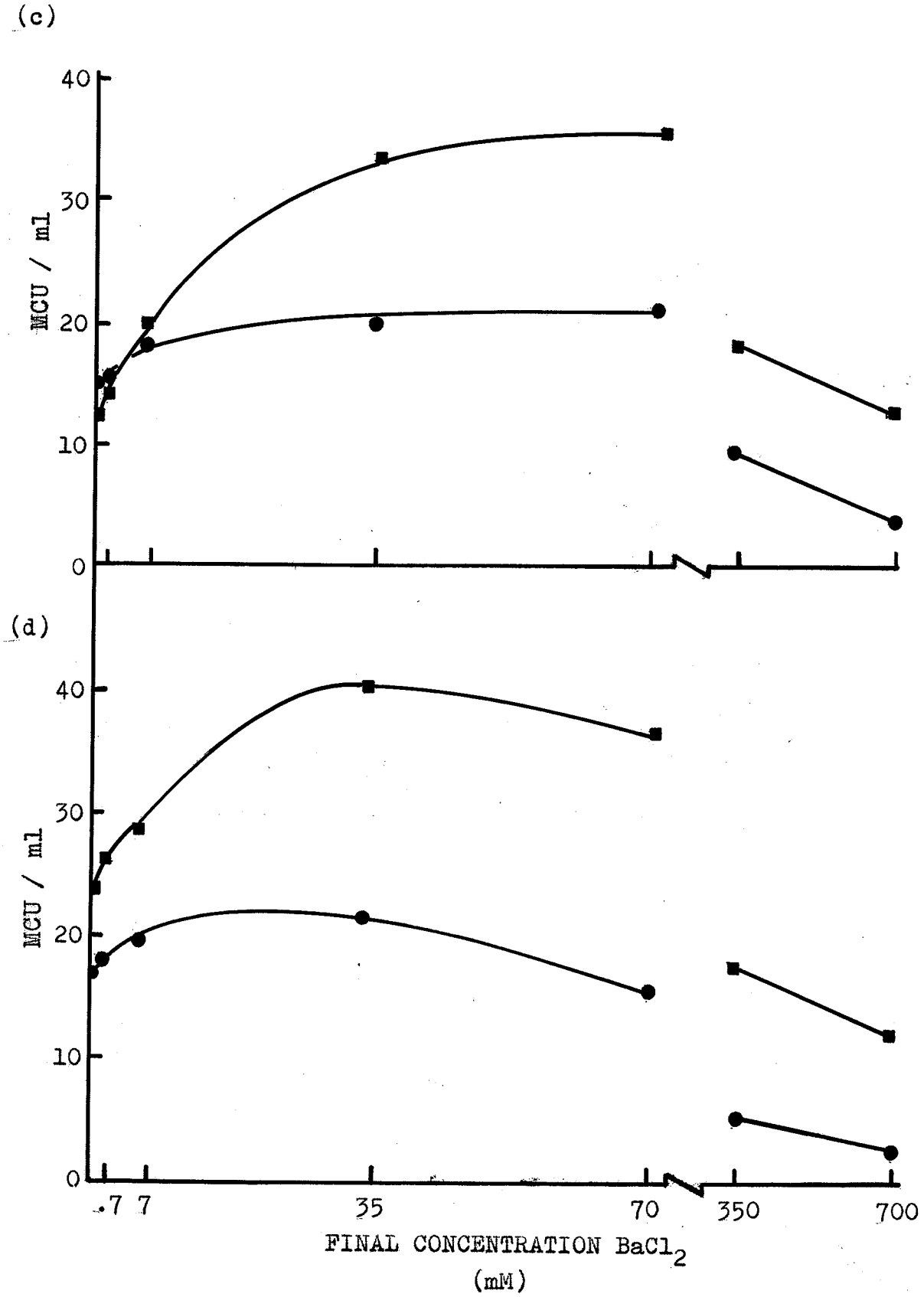

(c)
40
30
20
10
0
MCU / ml
(d)
40
30
20
10
0
MCU / ml
.7
7
35
70
350
700
FINAL CONCENTRATION $CaCl_2$ (mM)

Figure 14 Effect of different concentrations of barium chloride on milk-clotting activity of (—●—●—) T. vulgaris rennet and of (—■—■—) commercial calf rennet with different substrates - (a) Substrate 2A, (b) Substrate 2, (c) Substrate 1A, (d) Substrate 1.


(a)
40
30
20
10
0
MCU / ml
(b)
40
30
20
10
0
MCU / ml
.7
7
35
70
350
700
FINAL CONCENTRATION $BaCl_2$ (mM)


(c)
40
30
20
10
0
MCU / ml
(d)
40
30
20
10
0
MCU / ml
.7
7
35
70
350
700
FINAL CONCENTRATION $BaCl_2$
(mM)

Another general observation was that the degree of activation was dependent upon the salt and/or the presence of calcium chloride in the substrate.

With sodium chloride and potassium chloride, activation was maximal at 0.7 mM in the presence of calcium chloride and at 7 mM in its absence for the microbial rennet. With calf rennet activation was detected at 7 mM in only two of the substrates - 1A and 2. The presence or absence of calcium chloride was not as important a factor as for the microbial rennet. For the monovalent salts, the presence or absence of calcium chloride did not appear to affect the percent activation nor the degree of inhibition at higher concentrations for either enzyme. Calf rennet was more sensitive to higher concentrations (350 mM) of these salts than the microbial rennet.

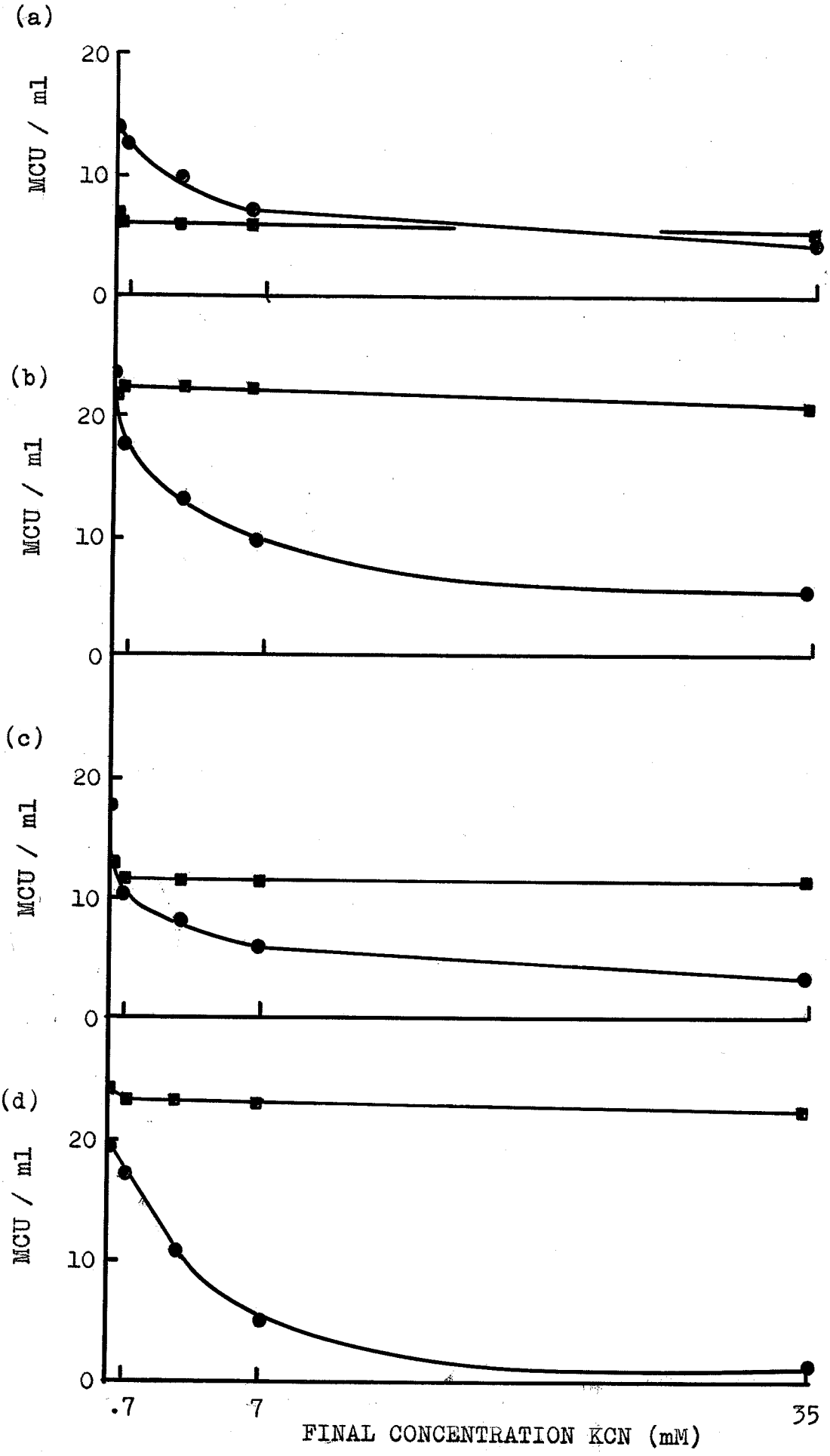
With calcium chloride and barium chloride, activation was maximal at 35 mM in the presence of calcium chloride and at 70 mM in its absence for both enzymes. The percent increase in activity was greater when there was no calcium chloride in the substrate (Table 9). Calf rennet was much more sensitive to these divalent salts. Although activity tended to decrease at higher concentrations (350 mM), for calf rennet activation was still evident for substrates 1A and 2A while for substrates 1 and 2 approximately 75% of the original activity was present. For the microbial rennet, approximately 70% of the original activity was detected in substrates 1A and 2A while in substrates 1 and 2, 50% of the activity remained.

Low concentrations of potassium cyanide (0.7 mM) had a detrimental effect on the microbial rennet (Figure 15). The degree of inhibition was similar in the presence or absence of calcium chloride. However on commercial calf rennet, potassium cyanide had no effect.

Table 9 The maximum percent increase in activity by different concentrations of calcium chloride and barium chloride with different substrates. Enzyme solutions without calcium chloride and barium chloride were used as controls in calculating the percent increase.

Concentration (mM)	Substrate	% Relative Activities	
		Calf Rennet	T. vulgaris Rennet
$CaCl_2$			
35	1	154	113
	2	171	114
70	1A	236	144
	2A	654	153
$BaCl_2$			
35	1	160	114
	2	178	115
70	1A	271	137
	2A	650	172

Figure 15 Effect of different concentrations of potassium cyanide on milk-clotting activity of (—●—●—) T. vulgaris rennet and of (—■—■—) commercial calf rennet with different substrates - (a) Substrate 2A, (b) Substrate 2, (c) Substrate 1A, (d) Substrate 1.


(a)
(b)
(c)
(d)
MCU / ml
20
10
0
.7
7
35
FINAL CONCENTRATION KCN (mM)

DISCUSSION

Approximately 112-fold purification of a milk coagulant produced by T. vulgaris was achieved by the procedure outlined, namely concentration by reverse osmosis, acetone precipitation and freeze-drying, dialysis and CM-cellulose chromatography. With DEAE-cellulose replacing CM-cellulose under similar conditions, the purification was 18-fold.

Reverse osmosis proved to be a valuable means of concentrating the supernatant as well as contributing to the overall purification scheme.

Separation of milk-clotting and proteolytic activities could not be demonstrated under the experimental conditions employed. Likewise, they have not been separated in commercial rennets from calves, M. miehei, M. pusillus and E. parasitica (Sardinas, 1972).

The decrease in the milk-clotting / proteolytic activity ratio after CM-cellulose chromatography may be due to the presence of sodium chloride which inhibits milk-clotting activity but not proteolytic activity. Independent studies with sodium chloride on a crude enzyme preparation indicated that at the theoretical maximum concentration of eluent (0.1 M NaCl), milk-clotting activity was inhibited. However, the effects of sodium chloride on proteolytic activity were not investigated.

As the pigmented material binds firmly to DEAE-cellulose at pH 7 and there is excellent recovery of activity with little change in the milk-clotting / proteolytic activity ratio, batch separation of pigment and enzyme could be achieved by mixing the dialysed acetone powder with precycled DEAE-cellulose, centrifuging, and washing the resin with a dilute salt solution (20

mM NaCl).

From T. vulgaris, only one milk-clotting fraction was isolated after CM-cellulose or DEAE-cellulose chromatography. This is not unusual as Arima (1971) isolated one active fraction from M. pusillus after repeated chromatography on Amberlite, DEAE-Sephadex and Sephadex G100. Sternberg (1971) found the majority of activity (51%) from M. miehei in one fraction after Amberlite and CM-cellulose chromatography. Whitaker (1970) isolated one active fraction from E. parasitica after repeated DEAE-cellulose chromatography.

Preliminary electrophoretic investigations not previously mentioned in detail included the use of the anionic system (pH 9.3) and the cationic system (pH 4.3) described in the Polyanalyst Instruction Manual (Buchler Instruments Inc., Fort Lee, New Jersey). Resolution in terms of protein bands was possible with the anionic system, but milk-clotting activity could not be detected after attempting to elute the enzyme fractions from the gel with acetate buffer, pH 5.6. Vamos-Vigyazo et al. (1973) reported similar results with the same technique at pH 8.3 on partially-purified and on purified preparations from calves, M. pusillus, and E. parasitica, and on pepsin. With the cationic system, no protein bands were detected for the T. vulgaris rennet after staining with amido black and Coomassie Brilliant Blue.

Two considerations in designing the electrophoretic system finally employed in this study were -

i) the similarity in stability between pH 5 and 7 for both enzymes

ii) the excellent correlation between protein bands and proteolytically-active zones achieved by Asato & Rand (1971) after vertical polyacrylamide gel electrophoresis at pH 7.15.

Another criterion was met by selecting pH 7.2 for the separation gel and pH 6.2 for the stacking gel. The pH of the stacking gel and the pH of the electrode buffers are usually the same, and one pH unit above or below the separation gel.

Phosphate buffer was selected in preference to tris buffer as the latter has been reported to have a detrimental effect on calf rennin at pH values above pH 6 (Vamos-Vigyazo et al., 1973).

The heterogeneity of calf rennin is well known. Usually two or three bands can be detected by electrophoresis. Asato & Rand (1971) after vertical polyacrylamide gel electrophoresis and Garnot et al. (1972) after starch-agarose electrophoresis reported two bands near the anode.

After vertical polyacrylamide gel electrophoresis, Shovers & Fossum (1972) reported a single active zone near the cathode for crystalline rennin. For commercial calf rennet, a small active zone near the anode and the crystalline rennin zone were detected. Pepsin exhibited the small active zone near the anode. Purified preparations from E. parasitica and M. miehei showed activity zones in the same region as the crystalline rennin. The M. pusillus rennet migrated to a position halfway between the crystalline rennin zone and the pepsin zone.

After paper electrophoresis (pH 6.6) of commercial preparations from calf rennet, E. parasitica and M. pusillus, a zone exhibiting milk-clotting and proteolytic activities was detected near the cathode for all preparations (Vamos & Morvai-Racz, 1970). Other inactive zones (two for calf rennet and one for each of the microbial rennet) were located at or near the origin.

Complete resolution was impossible for either one or all of the above enzyme preparations at pH 5.1, 8.6 and 8.0.

The method described in this study provided a good correlation between protein bands and proteolytically-active zones. Commercial calf rennet was resolved into three proteolytically-active zones but only two protein bands. Due to its width, one of the protein bands may account for two active components. Two proteolytically-active zones (R_f 0.690 and 0.762) were located near the front marker while the other (R_f 0.305) was near the beginning. For the T. vulgaris rennet, nine protein bands and three proteolytically-active zones were detected. Only one active zone (R_f 0.374) was in the vicinity of the slow-migrating band of calf rennet. Another active zone was nearer the beginning while the other was midway between the two rennet zones.

This procedure may represent a suitable method for the evaluation of new rennets for the following reasons. Other polyacrylamide gel electrophoretic techniques favor acid proteases either during the electrophoretic run (Shovers & Fossum, 1972) or by the method employed in detecting active zones (Asato & Rand, 1971). With the usual cationic and anionic systems for polyacrylamide disc electrophoresis, there is poor resolution and very poor or no recovery of milk-clotting and proteolytic activities. Another reason is that milk-coagulants are usually active in the pH region 5 to 7. Also, the results for calf rennet were comparable to published results.

Electrophoresis of other microbial rennets, especially the commercial ones, and of preparations of varying purity would be helpful in further evaluating the electrophoretic system described.

Differences in the effect of certain conditions on the milk-clotting properties of the T. vulgaris rennet and commercial calf rennet were evident.

Calf rennet was more sensitive to variations in substrate composition than the T. vulgaris rennet. For calf rennet, activity was lower in a substrate containing 50-50 milk-tris-maleate buffer compared to one containing 50-50 milk-acetate buffer. Both substrates contained 0.01 M calcium chloride. Also clotting time was longer for a substrate containing 50-50 milk-acetate buffer than a substrate containing undiluted whole milk. Calcium chloride was not added to either substrate. In both cases, there was no significant difference in coagulation time for the microbial rennet.

Tsugo & Yamauchi (1959) also reported a rapid increase in clotting time for calf rennin and pepsin when raw skim milk was diluted with distilled water below 70%. The microbial proteases from Aspergillus oryzae (partially-purified), Streptomyces griseus (crystallized), Bacillus subtilis (crystallized), and Pseudomonas myxogenes (crystallized), chymotrypsin and ficin exhibited little variation in clotting time down to 40% dilution.

There was very little difference in clotting time with a substrate containing 50-50 milk-acetate buffer plus 0.01 M $CaCl_2$ and a substrate containing undiluted whole milk plus 0.01 M $CaCl_2$ for the T. vulgaris rennet as well as for commercial calf rennet.

Milk-clotting activity was proportional to enzyme concentration (expressed as μg protein / ml) over a certain range for different preparations of the T. vulgaris rennet and for commercial calf rennet. The range depended on the purity and source of

enzyme. Within the limits of proportionality, the clotting strength (expressed as MCU / mg protein) was 33 times less for the dialysis residue and approximately 14 times less and 2.3 times less for the DEAE and CM fractions, respectively, compared to commercial calf rennet.

There was little or no clot formation above pH 7 for either enzyme. The rate of decline between pH 6.0 and 7.0, however, tended to be less for the T. vulgaris rennet than for the calf rennet. Lack of coagulation above pH 7 has been previously reported for calf rennet as well as for the three commercially available microbial rennets (i.e. from E. parasitica, M. pusillus and M. miehei) and milk-coagulants from C. consors, F. pinicola and I. lactus (Kawai, 1970; Arima, 1971; Sardinas, 1972). The loss in activity may be partially due to the increased stability of the calcium caseinate suspension at higher pH values (Schipper & Mulder, 1962).

The pH optimum of the above enzymes including T. vulgaris rennet is in the pH range 5.5 $\pm$ 0.2. In the indirect measurements of milk-coagulation two phases are important - the primary enzymatic phase and the secondary coagulation phase. Below pH 6.3, clotting tends to occur before the end of the primary phase at temperatures between 25°C and 40°C. As the pH approaches 6.7 at temperatures around 40°C or 6.3 at 25°C the clotting point moves towards the end of the primary phase (Foltman, 1959). At the other extreme, clotting occurs without the addition of enzyme at pH 5.0. The pH optimum is therefore dependent upon the rate of enzymatic activity and the effect of pH on the clotting point.

The pH stability range for the T. vulgaris rennet was 5 to 11 after a two-hour exposure at ambient temperatures while the range was 4.5 to 6.5 for commercial calf rennet for the same time period. The pH stability range reported is 5.3 to 6.3

(48 hours at 25°C) for calf rennin, 4.0 to 5.5 (24 hours at 25° C) for E. parasitica rennet, 4.0 to 6.0 (10 minutes at 60°C) for M. pusillus rennet, and 2.0 to 6.0 (24 hours at 40°C) for M. miehei rennet (Foltman, 1959; Arima, 1971; Sardinas, 1968, 1972).

The T. vulgaris rennet possessed greater thermostability than commercial calf rennet. The T. vulgaris rennet lost 15% of its clotting activity after 2 hours at 45°C, 43% after 15 minutes at 55°C and 72% after 20 minutes at 60°C. Commercial calf rennet lost 14% on exposure to 45°C for 10 minutes and 70% on exposure to 55°C for the same time period, while the M. pusillus rennet lost 6% and 21% respectively (Iwaski et al., 1967). The E. parasitica rennet lost 90% activity after 20 hours at 45°C and was completely inactivated on exposure to 60°C for 5 minutes (Sardinas, 1968).

In solution, the T. vulgaris rennet lost all milk-clotting activity at 25°C but only 10% activity at -10°C and 10°C after 24 days. The acetone powder stored at -10°C was stable for at least a year. Calf rennet powder and suspensions of rennin crystals appear to be very stable when stored at temperatures below 5°C for an indefinite time period (Foltman, 1970). For the E. parasitica rennet there was a 40% loss of potency at 20° C within 10 hours but little loss for several days thereafter (Sardinas, 1968). The M. miehei rennet retained over 90% activity after 8 days at 38°C (Sardinas, 1972).

For both rennets, T. vulgaris and calf, sodium chloride and potassium chloride exhibited a similar pattern on milk-clotting activity - slight activation at very low concentrations followed by inhibition at higher concentrations. Activation was not so pronounced for the commercial calf rennet. It was detected only

in two substrates, one containing 100% milk with no additional calcium chloride and the other 50-50 milk-acetate buffer plus 0.01 M $CaCl_2$. For the T. vulgaris rennet, activation was evident in all four substrates. The presence of calcium chloride shifted the point of maximum activation.

Prolongation of clotting time was evident at 0.07 M sodium chloride and potassium chloride for both enzymes. Sardinas (1972) reported an increase in clotting time for commercial calf rennet by 0.1 M sodium chloride and by 0.2 M sodium chloride for the three commercially available microbial rennets.

Calf rennet was more sensitive to activation by calcium chloride and barium chloride and therefore more tolerant (less inhibited) at higher concentrations than the T. vulgaris rennet. Unlike the microbial rennet the degree of sensitivity for calf rennet was dependent on the composition of the substrate. Activation for both enzymes, however, was more pronounced in substrates without additional calcium chloride.

The effects of calcium chloride have been extensively studied. Concentrations as low as 1 mM are known to lower clotting times (Kawai, 1970). Sardinas (1968) reported that the clotting times of calf rennet and of E. parasitica rennet decreased with calcium chloride concentrations up to 40 mM, but then slowly increased as the calcium concentration increased. Similar results were reported for the M. pusillus rennet (Sanner & Kovacs-Proszt, 1973). In this study activation was maximal at 35 mM in the presence of 0.01 M calcium chloride and at 70 mM in its absence for both enzymes. Loss of activity was also evident at higher concentrations.

Pozar-Hajnal et al. (1970) tested the calcium sensitivity of

crude preparations and commercial preparations from calves, E. parasitica and M. pusillus. The highest sensitivity was shown by the two commercial microbial preparations. The difference between them was not significant. They proposed that calcium sensitivity was an inherent property of the enzyme. The lower the calcium content of the enzyme preparation the higher its sensitivity.

Contrary to the above, Sardinas (1972) reported that of the three commercially available microbial rennets and calf rennet, the M. pusillus rennet proved most sensitive.

The industrial use of calcium chloride to decrease coagulation time is limited as the salt adversely influences the water-binding ability and salt content of the cheese (Pozar-Hajnal et al., 1970; Arima, 1971).

The effects of barium chloride have not been as thoroughly investigated although it has been suggested to have activating properties similar to calcium chloride. Pyne & McGann (1962) found that a higher concentration of calcium ions was necessary for coagulation of rennet-treated calcium caseinate than rennet-treated barium caseinate.

Inhibition at higher salt concentrations may be due to interference with ionic forces or competitive binding to κ casein (Cheeseman, 1962; Sanner & Kovacs-Proszt, 1973).

Potassium cyanide was a strong inhibitor of the T. vulgaris rennet but had no effect on commercial calf rennet. This suggests differences in the nature of the active site and/or the mechanism of enzyme action. The active centre of calf rennin has been identified as serine (Babbar et al., 1965). However, Yu et al. (1973) reported histidine residues at the active centre

of calf rennin and M. pusillus rennin. The M. pusillus rennin and M. miehei rennin are similar in that neither are metal-dependent nor possess serine or sulfhydryl groups at the active site (Arima, 1971; Sardinas, 1972). Although the effects of metal, serine and sulfhydryl inhibitors on E. parasitica rennin have not been thoroughly investigated, the evidence so far indicates that it is similar to the above two microbial rennets (Whitaker, 1970).

No definite conclusion can be stated regarding the nature of the active centre of the T. vulgaris rennet, as cyanide is a non-specific inhibitor. Inhibition by cyanide may occur by one of the following mechanisms -

(a) combination with an essential metal in the enzyme
(b) removal of a metal from the enzyme to form an inactive complex
(c) combining with a carbonyl group in the enzyme, cofactor or prosthetic group or in the substrate
(d) acting as a reducing agent to break essential disulfide linkages in the enzyme

(Dixon & Webb, 1966).

Enzymes such as ficin or papain in which the presence of a sulfhydryl group is essential for activity have been reported to yield bitter cheese (Babbar et al., 1965).

Since certain properties of calf rennet (i.e. pH optimum, pH stability range, and sensitivity to milk dilution, calcium chloride and sodium chloride) by the thromboelastographic method described are comparable to results obtained by other methods (e.g. Soxhlet, Berridge), this method is reliable for examining the milk-clotting properties of rennets. One major problem is that the temperature of coagulation cannot be easily altered.

The method described in this study for determining proteolytic activity may not be sensitive enough as activity could not be detected for calf rennet above pH 6 with either hemoglobin or casein. The optimum at pH 3.0 agrees with reported values around pH 3.5 (Sardinas, 1969). Proteolytic activity on casein has been determined at pH 6.5 (Arima, 1971). Differences may be due to the type of casein used and the general method of assay which involved Folin's reagent. Sternberg (1971), from whom this method was adapted, did not determine the pH activity curve for calf rennet.

In comparing rennets it is common practice to calculate a milk-clotting / proteolytic activity ratio (Kawai, 1970; Kawai & Mukai, 1970; Puhan, 1971; Arima, 1971). The value obtained depended upon the general method of assay for both proteolytic and milk-clotting activities, especially the length of incubation time with the proteolytic substrate. For example, the ratio for Hansen's rennet powder has been reported as 12.5 (Kawai, 1970), 167.0 (Kawai & Mukai, 1970), 1225 (Puhan, 1971) and 7350 (Arima, 1971). Usually both activities are not determined at the same pH, although the pH of determination is generally in the pH range 6.0 to 6.5, as this range is critical in the manufacture of cheese.

In computing such a ratio for the milk-coagulant from T. vulgaris and for commercial calf rennet, the result was very dependent upon the pH at which proteolytic activity was determined. For example with data from Figure 5, the ratio (MCU/µg tyrosine) at pH 4.8 is 2.2 for T. vulgaris rennet and 3.1 for calf rennet, while at pH 5.6 it is 1.2 for the T. vulgaris rennet and 10.2 for calf rennet. As no activity could be detected above pH 6.0 for calf rennet, no ratio could be calculated. However, the ratio for T. vulgaris rennet progressively decreased until it

attained a minimum around pH 7 where proteolytic activity is optimal. Therefore in addition to the ratio, the degree of proteolytic activity in the milk-coagulant pH range 5 to 7 should be considered.

Proteases may be classified as acid, neutral or alkaline depending on the optimum pH for proteolytic activity. Acid proteases exhibit maximum activity on hemoglobin at pH 4 $\pm$ 2.0. Neutral proteases are active on casein over the range pH 5 to 8, peaking at 7.0. Maximal activity for alkaline proteases occurs at pH 8.5 $\pm$ 1.0.

As the enzyme from T. vulgaris exhibited maximal activity on casein and hemoglobin around pH 7, it may be considered to be a neutral protease. The three commercial microbial rennets and calf rennet are acid proteases (Sardinas, 1969). The recommended rennet substitute system from B. subtilis contains an acid protease and a neutral protease (Murray & Kendall, 1972). The coagulum formed by the B. subtilis neutral protease was softer, more ashy and of less rubbery texture than that produced by calf rennet. However, together with the acid protease which did not coagulate milk, a coagulum of a better overall quality was produced.

Certain information gained on the milk-clotting activity and proteolytic activity of the T. vulgaris rennet in the model systems may be summarized in two categories - properties favoring the manufacture of cheese and those against it. On the plus side, the microbial rennet is less influenced by dilution of milk, slightly more active and stable over the pH range 5.6 to 7 and more thermostable than calf rennet. Although it is not as sensitive as calf rennet, it is activated by minute concentrations of calcium chloride and barium chloride. The

lower sensitivity to calcium may be important as pH and calcium concentrations in milk vary with season and geography. Both rennets exhibited similar patterns for varying concentrations of sodium chloride and potassium chloride. A colorless and odorless preparation with approximately one-eighth the clotting strength of calf rennet is possible with DEAE-cellulose chromatography. With CM-cellulose, the same general preparation is possible but there is a greater loss of activity and the milk-clotting / proteolytic activity ratio is much lower. On the negative side, the T. vulgaris rennet ia a neutral protease and therefore there is a high degree of proteolytic activity in the milk-coagulation pH range 5 to 7. Dissimilarities also exist in the electrophoretic pattern of the two rennets. However, one proteolytically-active band of the microbial rennet was in the vicinity of an active rennet band. As potassium cyanide strongly inhibited the T. vulgaris rennet but had no effect on calf rennet there is a strong possibility that the active site and/or the enzyme clotting mechanism of the two rennets are different. On the basis of results reported in this study and on the inability of commercial microbial rennets from M. pusillus, M. miehei and E. parasitica, which are more similar to calf rennet than the T. vulgaris rennet, to produce cheeses equal in quality to cheeses produced with calf rennet, it seems unlikely that the T. vulgaris rennet will produce cheese equal in quality to calf rennet.

Gupta & Pereira (1974) reported the satisfactory production of cheddar cheese after ripening for 11 to 16 weeks at 7°C. The mealiness and slightly acid taste in the case of the microbial rennet was attributed to imperfection in the technique (i.e. improper acid control during the manufacturing process). Neither defect was reported for cheese from calf rennet prepared under

the same conditions. On the basis of this study, these defects may be due to the biochemical properties of the microbial rennet (e.g. the high degree of proteolytic activity in the pH range 5 to 7). There was no significant difference between the cheeses produced by the two rennets in terms of yield, fat content and moisture. Therefore in spite of differences in biochemical properties between the microbial rennet and the calf rennet, the T. vulgaris rennet may be suitable for the production of some cheeses.

SUMMARY

1. Due to the properties of the T. vulgaris rennet and/or the nature of the supernatant containing the enzyme, ammonium sulfate precipitation, heat treatment at 55 C for 10 minutes and precipitation at pH 5 were unsatisfactory for concentrating and/or purifying the enzyme. Reverse osmosis and acetone precipitation, however, were valuable techniques for the above purposes.

2. The fold-purification was approximately 112 after CM-cellulose chromatography but approximately 18 after DEAE-cellulose chromatography under similar conditions.

3. With DEAE-cellulose chromatography, approximately 40% of the original activity was recovered while with CM-cellulose chromatography, approximately 24% of the original activity was recovered.

4. The milk-clotting / proteolytic activity ratio remained relatively constant after DEAE-cellulose chromatography but decreased approximately five-fold after CM-cellulose chromatography.

5. Proteolytic activity was evident from pH 4 to 10, peaking at pH 7 for the T. vulgaris rennet and from pH 3 to 5, peaking at pH 3 for commercial calf rennet.

6. For the T. vulgaris rennet, proteolytic activity was greater in substrates prepared with boric acid - borax buffer than ones prepared with citrate phosphate buffer or phosphate buffer.

7. The T. vulgaris rennet and commercial calf rennet exhibited milk-clotting activity over the pH range 5.5 to 7 with an optimum at pH 5.6.

8. The pH stability range after two-hour exposure at ambient temperature was 5 to 11 for the T. vulgaris rennet and 3 to 7 for commercial calf rennet.

9. In solution, the T. vulgaris rennet lost all milk-clotting activity after 5 minutes at 65°C and after 24 days at 25°C. The acetone powder, stored at -10°C was stable for at least a year.

10. The clotting strength of the T. vulgaris rennet was lower than that of commercial calf rennet. The degree dependent upon the purity of the enzyme.

11. Commercial calf rennet was more sensitive to substrate composition, to milk-dilution and to activation by barium chloride and calcium chloride than the T. vulgaris rennet.

12. For both enzymes, slight activation was evident at very low concentrations of sodium chloride and potassium chloride.

13. High concentrations of the chloride salts inhibited the milk-clotting activity of both enzymes.

14. Potassium cyanide strongly inhibited the milk-clotting activity of the T. vulgaris rennet but had little or no effect on commercial calf rennet.

15. The electrophoretic technique described provided good correlation between protein bands and proteolytically-active zones. Both enzymes revealed three proteolytically-active zones. However, only one microbial rennet proteolytically active zone possessed an R_f value similar to an active zone of calf rennet.

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