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Proteolytic and coagulating enzymes of enterococci

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NOTE TO USERS

PROTEOLYTIC AND COAGULATING ENZYMES OF ENTEROCOCCI

by

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INTRODUCTION

Several streptococci commonly encountered in milk are known to bring about coagulation and breakdown of milk proteins. Most of these organisms also have the ability to act at acid reaction, and the term "acidoproteolytic" commonly is applied to them. This breakdown is believed to be brought about by two types of enzymes, the exocellular proteinases and the endocellular peptidases; the former change the whole protein into polypeptides, at which stage the latter enzymes take over and complete the splitting to the amino acid stage, thus providing some of the nutritional requirements of the bacterial cell. The role of the sweet-curdling or what is often termed a rennin-like enzyme is not clearly understood.

In spite of the relative importance of the organisms responsible for these changes in a variety of dairy products, no detailed studies on the enzyme systems responsible have been reported.

Streptococcus liquefaciens, which forms the subject matter for the present investigation, is very commonly found in milk. The organism is resistant to normal pasteurization and carries over in the pasteurized milk used for cheese making and other purposes. It also is a cause of

rapid coagulation of milk, which has been assumed to be due to a rennet-like enzyme, and brings about rather rapid proteolysis in milk with a subsequent production of bitterness.

With a view to gaining a better understanding of the role of this organism, the study of its enzymatic processes as they affect milk proteins was undertaken.

STATEMENT OF PROBLEM

The purpose of the investigation was to study the conditions affecting the production of proteolytic and coagulating enzymes by Streptococcus liquefaciens and to determine the characteristics of these enzymes.

REVIEW OF LITERATURE

In recent years, a great deal of information has accumulated regarding the growth requirements of microorganisms in general and lactic acid bacteria in particular. Several excellent reviews, among them those by Snell (1948, 1949), have appeared on this important topic. Therefore, no attempt will be made to present a detailed discussion of this material here. However, attention will be given to the work reported on the relationship of nutrients in the growth media as they affect the production of proteolytic and coagulating enzymes by bacteria.

Nutrition in Relation to Enzyme Production

Brunton and Macfadyen (1889) grew Koch's comma spirillum (Vibrio comma) and a few other organisms in meat broth for several days. They found that heating the growth medium to 60-70° C. killed the bacteria, but the heated growth medium still was capable of liquefying gelatin.

However, Fermi (1890, 1891) seems to be the first one to demonstrate conclusively that an active proteolytic enzyme can be obtained from a growth medium. He accomplished this by growing Micrococcus prodigiosus (Serratia marcescens)

and Bacillus pyocyaneus (Pseudomonas aeruginosa) and passing the growth media through Chamberland filters to get cell-free extracts. While gelatin and fibrin were hydrolyzed by these filtrates, egg-albumin and blood serum were found resistant to attack. It is of interest to note that he failed to obtain any proteolytic activity when the organisms were grown in protein-free, purely inorganic media.

In continuation of his earlier work, Fermi (1892), using the same test organisms, grew them on an inorganic medium containing ammonium phosphate, potassium phosphate, magnesium phosphate and either glycerine or cane sugar as a source of carbon and found a gelatin liquefying enzyme.

Gorini (1893) reported production of a rennet-like enzyme by Bacillus prodigiosus (S. marcescens). This enzyme was produced not only in milk but also in diverse types of media such as broth, gelatin, agar or potato and also in the presence of casein and lactose.

In 1907, Nicolle, using Bacillus subtilis, showed that when gelatin, coagulated serum or egg-albumin was present in the medium, the organism produced enzyme active against the corresponding protein. The enzyme also had a "clearing" effect on milk. However, when the organism was grown in nutrient broth, the filtrate could attack gelatin only. This worker postulated the formation of a trypsin and a gelatinase by this organism.

Macfadyen (1892) believed that in a highly nutrient medium like gelatine-peptone, very little proteolytic enzyme was produced. This, he thought, was due to the presence of enough readily assimilable nutrients, making it unnecessary for the organisms to have any "struggle for existence".

However, Drummond (1914) was of the view that protease could be formed from nothing less complex than a peptone and hypothesized formation of a complex, preliminary compound of enzyme and peptone which then proceeded to activate the enzyme for further action.

Employing B. pyocyaneus (Ps. aeruginosa) and B. prodigiosus (S. marcescens) as the test organisms, Diehl (1919) failed to obtain any protease on an inorganic medium, in spite of good growth of the organisms. He reported that amino acids were adequate sources for enzyme production. It is interesting to note that he reported the production of a specific gelatinase but no caseinase when glycine was the nitrogen source, while the reverse was the case when tyrosine was substituted for glycine in the medium. This is of particular interest in view of the fact that glycine is abundant in gelatin, whereas casein is a good source of tyrosine (Schmidt, 1944).

These results have been questioned by Wilson (1930), who failed to duplicate the findings of Diehl. Using a basal inorganic medium and B. subtilis and Ps. pyocyaneus,

this worker studied the effect of addition of several compounds to the basal medium on the enzyme production. Addition of lactate, glycerol, acetate, tartarate and glucose resulted in enzyme production in all cases when the cultures were incubated 3-7 days. The activity was measured by gelatin liquefaction and casein opalescence tests. When tested as the sole nitrogen source, alanine supported greater enzyme formation than did tyrosine. The results for both the organisms were identical. Addition of calcium or magensium ions to the medium did not result in increased enzyme formation. No relationship was found between the number of cells and the quantity of enzyme production. Protease formation by the organisms studied was believed to be a normal product of cell metabolism, independent of the composition of the medium, except as the ingredients stimulated or inhibited cell growth.

In similar studies on Pseudomonas putrefaciens and Bacillus mesentericus (B. subtilis), Merrill and Clark (1928) found that gelatinase was produced in a simple inorganic medium. These workers came to the conclusion that growth, as evidenced by bacterial numbers, does not account for very significant differences in protease formation, as compared to the variations which occur as a result of the presence or absence of specific inorganic ions in the medium or the availability of oxygen to the growing bacteria.

The effect of several growth media on the production of rennin by B. prodigiosus (S. marcescens) has been studied by Wahlin (1928). He found that the enzyme was produced irrespective of whether the source of nitrogen in the medium was organic or inroganic.

The results of these latter workers have been supported by the work of Haines (1931), who obtained protease formation by B. mesentericus (B. subtilis) and an unidentified Pseudomonas, when grown on an inorganic medium. Both gelatin and casein were attacked by the enzyme. Addition of calcium and magnesium salts to the medium resulted in increased enzyme formation. The addition of these salts also stimulated the growth.

Extending his earlier work, Haines (1934) reported that a Pseudomonas and a Proteus under examination produced a gelatinase irrespective of the type of medium, so long as sufficient bacterial growth occurred. Addition of calcium and magnesium ions greatly enhanced the enzyme production.

Console and Rahn (1938), working with gelatinase production by B. subtilis, found that addition of 0.5 per cent glucose to the broth almost completely prevented the proteolytic activity.

Kocholaty, Weil and Smith (1938) grew Clostridium histolyticum in three different media containing neopeptone, horse meat and casein and found that the proteinase produced had maximum stability in the casein medium.

In continuation of their earlier work, Kocholaty and Weil (1938) observed that pH of optimum activity on gelatin by the proteinase of the same organism dropped from pH 7.0 to pH 6.7, when the initial reaction of the growth medium was lowered from pH 7.0 to pH 6.0. In addition to this, when the final pH in a 3 per cent casein medium was allowed to drop from pH 7.4 to pH 5.8 by addition of 1 per cent glucose to the medium, the reaction for optimum activity of the enzyme was lowered from pH 7.0 to pH 6.0.

Using four thermophilic rods, Chopra (1945) found that addition of various carbohydrates in a concentration of 2 per cent to the nutrient broth reduced their proteinase production very considerably. He stated the extent of their proteinase-retarding effect in the following descending order: starch, dextrin, glycerol, sucrose, dextrose and levulose.

There is no reference in the literature regarding the influence of growth media on the protease production by enterococci, the group to which Streptococcus liquefaciens belongs. However, Woolley and Hutchings (1940), first reported the growth of a strain of Streptococcus zymogenes, a closely related organism, on a chemically defined medium. This medium contained riboflavin, pantothenic acid, pyridoxine and seven amino acids, in addition to glucose, inorganic salts, buffer salt and a reducing agent.

Schuman and Farrell (1941) found that Streptococcus faecalis, except for some differences in amino acid requirements, had the same requirements as were reported by Woolley and Hutchings.

In a rather detailed investigation of the growth requirements of the enterococcus group of organisms, Niven and Sherman (1944) examined nineteen strains, representing members of all the four species of this group. Growth of their organisms was not noted on the medium reported by Woolley and Hutchings, a situation ascribed to the probable impurity of compounds employed by those earlier workers. Niven and Sherman found that all of the nineteen strains examined, including four strains of S. liquefaciens, required pantothenic acid, nicotinic acid, pyridoxine and biotin. Riboflavin was required by seventeen of the cultures, while only seven seemed to require folic acid. None of the strains examined required thiamine. Out of these nineteen cultures, fourteen could grow in a medium containing thirteen amino acids. Purine and pyrimidine bases, although not essential, stimulated growth. These workers therefore came to the conclusion that there was no significant difference in the general nutritional requirements among the members of the enterococcus group.

It is quite evident that there is a considerable contradiction and confusion in the literature with regard

to the influence a growth medium exerts on protease production by the bacterial cells. The results reported by various workers on this subject range from the indispensability of protein or at least a peptone in the growth medium for the production of proteases by the organisms to the production of these enzymes in simple media with inorganic salts as the only source of nitrogen. Such divergent observations, however, are to be expected in view of the great differences in the bacterial species employed in these studies and relatively little attention that has been paid to the various conditions that affect the production of bacterial enzymes.

Production of Rennet-like Enzymes by Bacteria

Warrington (1889) probably was the first to report on the production of a rennet-like enzyme by organisms. He demonstrated the production of such an enzyme by Bacillus fluorescens liquefaciens (Pseudomonas fluorescens). He grew the organism in milk.

Conn (1892) showed considerable interest on this subject. He studied seven organisms, common in dairy products. In addition to causing sweet curdling of milk, all of these organisms liquefied gelatin and produced a coagulating enzyme. However, the various species differed considerably in their gelatinolytic and coagulating abilities. When

grown on a beef peptone bouillon, there was only a very slight coagulating activity; no mention was made of the corresponding effect on proteolytic power. Conn also reported greater enzyme production when the organisms were incubated at temperatures below 35° C. In one case, after four months of incubation in milk, the coagulation power of the organism was lost, while the digestive activity still remained.

Using Bacillus butyricus (Bacillus cereus), Hueppe (1884) showed that the organism produced coagulation of milk, followed by subsequent dissolution of the coagulum. The end-products of digestion were found to include tyrosine, leucine and ammonia. Proteolysis occurred at an alkaline reaction. He held the view that the coagulating and proteolytic activities might be the same, although he noticed suppression of coagulating activity on addition of small amounts of acid such as carbolic acid.

Gorini (1893) reported coagulating and proteolytic activity in the milk in which B. prodigiosus (S. marcescens) was grown. The supernatant liquid showed a higher coagulating activity when the organism was grown at 20° C., while increased proteolytic power was demonstrable when the cultures were incubated at 37° C. This has been explained on the basis of greater interaction of casein and rennet than that between the protease and casein at higher temperatures of incubation.

In 1904, Hata reported that B. prodigiosus (S. marcescens) and B. fluorescens liquefaciens (Ps. fluorescens) grown in milk produced an albumin- and casein-coagulating enzyme. The enzyme was purified from the milk filtrates by precipitation with ammonium sulphide and alcohol. He estimated that 1.0 g. of such a preparation yielded 28,000 trypsin units and 150,000 rennet units (the units used were purely arbitrary) in case of B. prodigiosus (S. marcescens) and 90,000 trypsin and 380,000 rennet units for B. fluorscens liquefaciens (B. fluorescens). Significantly, the ratio between proteolytic and coagulating activity of his preparation was approximately 1:5 for both organisms.

Wahlin (1928), using a strain of B. prodigiosus (S. marcescens), obtained a rennin-like enzyme, irrespective of the nature of the nitrogen source of the medium. This, it might be pointed out, contradicts earlier work of Diehl, who failed to obtain either gelatinase or caseinase in media devoid of complex nitrogenous organic compounds. This worker obtained higher rennin production when the cultures were incubated at 20° C., rather than at 37° C. Clotting of milk was found to be independent of the bacterial cell content of the test material. He further reported that in comparison with animal rennin, his enzyme preparation coagulated heated milk more readily.

It will thus be seen that production of a rennet-like

enzyme by bacteria has been reported by several investigators. Significantly, all of the organisms reported on by the various workers also are the organisms known for their proteolytic activity. However, such activity is influenced by the individual characteristics of the test organisms in respect to their conditions of growth, pH, temperature and the substrates employed for testing their activity. It also might be pointed out that none of the workers so far has been able to conclusively separate the coagulating fraction from the proteolytic fraction.

Proteinases Produced by Bacteria

Proteinase is a term first employed by Willstatter to designate enzymes attacking whole proteins.

Mesernitzky (1910) studied the decomposition of gelatin by M. prodigiosus (S. marcescens). He grew the organism in broth and after growth, passed the medium through a Chamberland candle to obtain a cell-free preparation. Gelatin breakdown was followed by a chemical method. He concluded that secondary products, precipitable with tannin, were formed first, followed by peptides and a simple product which he found to be glycine.

In 1918, Corper and Sweany, adding toluene or chloroform to broth cultures of tubercle bacilli to stop their

growth, isolated three different types of enzymes: a trypsin-like enzyme which split proteins in an alkaline reaction, an erepsin-like enzyme acting on peptones in acid medium, and a weak pepsin-like fraction which broke down proteins at acid reaction of the substrate.

Blanc and Pozerski (1920) made detailed studies on two strict anaerobes, Bacillus sporogenes (Clostridium sporogenes) and Bacillus histolyticus (Cl. histolyticum). They grew the cultures on a broth made from putrid meat with added calcium sulphide at pH 7.0. They made both cell-free broth cultures and cultures as such without any filtration to remove the cells. Gelatin was digested very readily, while the digestion of coagulated ovalbumin and serum proteins was very slow. A rennet-like enzyme also was formed which coagulated fresh milk but acted on heated milk only when calcium was added to the milk. This, it might be pointed out, does not agree with the later work of Wahlin (1928), where heated milk was coagulated more readily, although a different organism was used by this latter worker. Blanc and Pozerski also report that their filtrates in addition contained an active casease which was inhibited by the presence of calcium.

Weil and Kocholaty (1937) have investigated in great detail the proteinases formed by Cl. histolyticum. These proteinases were found to be active at pH 7.0 with gelatin

as a substrate. These enzymes were activated considerably by the addition of sulphydryl compounds and several metallic ions. The activation was particularly pronounced when cysteine-iron combination was used. Later, Weil, Kocholaty and Smith (1939) extended their work to include several aerobic and other anaerobic species. The results obtained were in conformity with their earlier findings.

Isolation of a papain-like enzyme has been reported by Elliott (1945) from two strains, K-43 and T1/79, belonging to Group A of the hemolytic streptococci. This worker purified the enzyme by alternate use of alcohol and ammonium sulphate. The enzyme was activated by several reducing agents and was inhibited by iodoacetic acid. Proteinase production by B. subtilis on a peptone broth has been reported by Stockton and Wyss (1946). Aeration of the growth medium and addition of several inorganic salts enhanced the enzyme production.

From the reports reviewed here, it will be recognized that proteinases are quite widespread among bacteria. It now is generally agreed that these enzymes are extracellular in character. They also usually appear to have their optimum activity at an alkaline reaction of the substrate. A number of these proteinases, especially those produced by anaerobes or facultative anaerobes, appear to be activated by several reducing agents. Some of them appear to

be activated by a variety of metallic ions.

Proteinases of S. liquefaciens

In 1894, von Freudenreich isolated an organism from bitter cheese and termed it Micrococcus casei amari (S. liquefaciens). He reported the morphological and biochemical characteristics of this organism and found it could liquefy gelatin rapidly. The organism could also digest milk after coagulating it. After growth for 24 hours at 37° C., the milk substrate was coagulated and bitter, the latter becoming quite pronounced after 48 hours. At 20° C., however, the coagulation and bitterness in milk occurred after 48 hours of incubation.

Using several unidentified cocci from milk and from cow's udders, Gorini (1902) observed that proteolysis was distinct from the acid production. The organisms examined brought about proteolysis even in acid reaction of the medium. The truth of his earlier findings has been amply substantiated by the work of several workers on the bacterial peptidases.

In a study of several cultures liquefying gelatin and causing proteolysis of milk, Orla Jensen (1919) isolated several strains and designated them S. liquefaciens. He found his cultures identical with the one first isolated and described by von Freudenreich.

Frazier (1928), using five strains of S. liquefaciens, found that this organism in milk produced ammonia, liberated tryptophan, as determined by the bromine test, and also released considerable amount of amino nitrogen, as shown by the formol titration method. In a comparison of steamed and autoclaved milk as a growth medium for these organisms, the latter favored the action of more actively proteolytic strains.

Gorini and Gorini (1935) reported that old milk cultures of Mammococcus Gorini (S. liquefaciens) possessed less proteolytic activity than the young cultures. The maximum stability of the proteases was found to be around pH 7.0. The enzyme was rapidly destroyed at pH 4.3 or below. However, on gelatin substrate, maximum activity was found at pH 5.5, using phosphate buffer, and pH 6.1 when phosphate-citrate buffer was used. There was increased proteolytic activity in the latter case, which was considered to be due to the activation of the enzyme by citrate.

In a study of 101 cultures of S. liquefaciens, Long and Hammer (1936) determined, among other things, the protein breakdown in skim milk by four representative strains. The organisms were grown for different intervals at 21 and 37° C. The milk serum was analyzed for total nitrogen and nitrogen soluble and insoluble in trichloroacetic acid, ethyl alcohol and phosphotungstic acid. Considerable

increases in soluble nitrogen were recorded. However, no significant differences were obtained between the different strains. Longer incubation periods at either incubation temperature did not increase the protein breakdown to any appreciable extent. The effect of incubation temperature was variable. The distribution of nitrogen in the various fractions was found to be independent of the organisms used and the incubation temperature employed.

Gorini (1942) studied proteinase production by "acido-proteolytic" Mammococcus Gorini (S. liquefaciens). When grown in milk, the organism showed maximum enzyme production on 15-20 hours incubation at 37° C., or 40 hours at 20° C. Destruction of the enzyme occurred at 100° C. Maximum stability of the enzyme was reported to be from pH 7.0-9.0. It is of interest to note that throughout his work this investigator measured the proteinase activity at pH 6.5, using gelatin as a substrate, despite his earlier report (Gorini and Gorini, 1935) stating an optimum pH of 5.5 using phosphate buffer.

Peptidases Formed by Bacteria

Considerable work has been reported on the endocellular peptidases of bacteria. However, in most of the earlier reports, filtrates of growth media have been used as a source of these enzymes. Later work has revealed that the

peptidases, in most instances, are not released in the growth medium until after 24 hours incubation of the cultures. In those instances where intact cells were used, the results obtained were not very conclusive.

Gorbach (1937), in his work on Caseococcus Gorini (S. liquefaciens) employing leucylglycine as a substrate, found two optima, one at pH 4.8 and the other at pH 7.0-8.4. An incubation period of five days was used for the determination of enzyme activity.

Berger, Johnson and Petersen (1938a) probably were the first ones to study systematically the bacterial peptidases. In a well-controlled study of Leuconostoc mesnteroides, these workers used autolyzed cell preparations. Using several di- and tri-peptides as substrates, they found that the peptidases of the organism were able to split both the D- and L- components of the racemic peptides. Hydrolysis was activated by several metal ions, such as Zn, Pb, Cu, Mn, Sn, Cd and Hg. The peptidases were found to possess two pH optima for activity, one around pH 5.0-6.0 and the other at pH 7.0-8.0. Their studies thus substantiate the earlier findings of Gorbach on another organism. These same workers (1938b) made a rather extensive survey of peptidases of twelve bacterial species. Cell-free extracts of Escherichia coli, Bacillus megatherium, Proteus vulgaris, Ps. fluorescens, B. mesentericus (B.

subtilis), B. subtilis, Clostridium butylicum, Cl. sporogenes, Clostridium acetohistolyticum, Lactobacillus pentosus, Propionibacterium pentosaceum and Phytomonas tumefaciens were used. In most cases, peptidases were active in the pH range of 8.0-9.0. However, L. pentosus and P. pentosaceum had their optimum activity at pH 5.0-6.0. The activity of the preparations was not impaired at pH 8.0 when held at 40° C. for incubation periods up to 48 hours.

Metal activation of peptidase preparations of P. tumefaciens, B. megatherium and P. vulgaris was studied by Berger and Johnson (1939). Leucylpeptidase of P. vulgaris was activated by Mn and Mg ions. Hydrolysis of di-glycine by peptidases of B. megatherium also was activated by these ions.

Berger, Johnson and Bauman (1941) studied the effect of D-peptidases from B. megatherium, L. mesenteroides and a few other organisms. All of the organisms seemed to possess D-peptidases active against D-leucylglycine, the rate of hydrolysis being different for various species. Most of these preparations were activated by Mn or Co ions or with cysteine in combination with these two metal ions. Optimum pH for D-peptidase of L. mesenteroides was found to be between pH 7.0-8.0.

Gorini, Grassman and Schleich (1932), using peptidases of Enterococcus Gorini (Streptococcus faecalis), found an

optimum pH of 7.8 using leucylglycine as a substrate and an optimum pH of 7.0 with leucyldi-glycine for polypeptidase activity.

Using some "acidoproteolytes", Gorini (1942) has reported that peptidases from these organisms have an optimum at pH 8.0, using alanylglycine as the substrate. Interestingly, this worker used an incubation period of eight days for determination of peptidase activity. Destruction of the dipeptidase required 20 minutes at 80-100° C. In comparison to this, the proteinases and the polypeptidases were destroyed instantly at 100° C.

From the foregoing discussion it would seem that the bacterial peptidases are active in both acid and alkaline reaction and some organisms appear to possess D-peptidases.

EXPERIMENTAL METHODS

Designations and Sources of the Cultures and Their Propagation

Of the nine cultures used in this study, four (7, 19, 20 and C) were obtained from the collection of the Dairy Bacteriology Laboratories at Iowa State College. Strain B was obtained from the collection of Dr. M. P. Baker. Strains O, Y, E and N were isolated from different sources by members of a class in the identification of dairy organisms.

Throughout this investigation, the stock cultures were carried in litmus milk with transfers at approximately ten-day intervals. Before testing for proteolytic and coagulating activities, the organisms were transferred three or four times at daily intervals. Unless otherwise stated, all the cultures were incubated for 24 hours at 35° C.

Preparation of Media

Niven and Sherman's medium

The simplified amino acid medium of Niven and Sherman (1944) was used with certain modifications. K_2HPO_4 was

increased from 0.5 to 1.0 g. per 100 ml. and 0.5 g. glucose was replaced by 0.2 g. lactose. This was necessitated in view of the preliminary trials indicating substantial inactivation of the enzyme system under investigation at low pH levels, which resulted when the various strains were grown in the unaltered Niven and Sherman's medium. In addition to this, thiamine (5γ per 5 ml.) also was included in this medium. The reasons for this would be described in the section under Experimental Results.

Stock solutions for making the synthetic medium were kept in brown bottles in a cooler at 2-5° C. Toluene in a thin layer was added as a preservative to all the stock solutions.

Preparation of vitamin-test casein medium

Five g. of vitamin-test casein (General Biochemicals) was suspended in 100 ml. of 0.05 N NaOH and steamed for 10-12 minutes. For each 100 ml. of the medium to be made up, 40.0 ml. of this five per cent casein solution were taken and 40.0 ml. of distilled water added. KH_2PO_4 (1 per cent) and lactose (0.2 per cent) were included at this stage and the pH was adjusted to approximately 6.8, using 1.0 N NaOH. The six vitamins then were added (1.0 ml. of each stock solution for 100 ml. of the medium). The medium was made up to 100 ml. with distilled water, dispensed in

5 ml. quantities in test tubes and sterilized at 15 lb. pressure for 13 minutes.

Composition of the vitamin-test casein medium as used was:

	per 100 ml.
Vitamin-test casein.	2.0 g.
Riboflavin.	100 γ
Calcium pantothenate	100 γ
Nicotinic acid	100 γ
Pyridoxine.HCl	100 γ
Biotin	0.1 γ
Folic acid	1.0 γ
KH_2PO_4	1.0 g.
Lactose.	0.2 g.

Preparation of casein medium

Casein (Fischer Scientific Co.) was dissolved in NaOH solution in the same way as the vitamin-test casein was. No vitamins were added to this medium, since the casein used was prepared by precipitation from milk and would be expected to contain some of the B-complex vitamins normally present in milk.

The casein medium contained 2 per cent casein, 1 per cent KH_2PO_4 and 0.2 per cent lactose.

Methods for Measurement of Proteolysis Due to Extracellular Proteinase Activity

Preparation of the casein substrate

All the determinations reported were made on a 1.0 per cent casein substrate. In preparing this medium, 3.0 g. of washed casein (Fischer Scientific Co.) were suspended in 100 ml. of 0.05 N NaOH, steamed for 10-12 minutes in a steamer and 30.0 ml. of 0.5 N KH_2PO_4 solution added. Merthiolate solution (2.5 mg. per ml.) was added to bring its effective concentration to 1.0 mg. per 40 ml. of the substrate, in order to prevent possible bacterial growth during incubation period. To the above mixture, 150 ml. of distilled water then were added. The pH was adjusted to 7.4, using 1.0 N NaOH and the volume made up to 300 ml. The substrate thus prepared was allowed to stand overnight in the cooler to equilibrated and the pH checked again before use.

All the proteinase determinations were made at pH 7.4, since that was found to be the level of optimum activity. For convenience the data on optimum pH for casein digestion have been presented in Figure 2.

Enzyme test material

Organisms were grown throughout at 35° C. in the various

culture media and centrifuged at the end of the incubation period, using a speed of 1,300 r.p.m. for 12-15 minutes. The supernatant material was used for proteinase and coagulation tests.

Determination of enzyme activity

For each determination, 5.0 ml. of the 1.0 per cent casein substrate were taken in a large test tube and allowed to come to the water bath temperature of 37.5° C. (±0.02° C.). To each of the tubes, 0.2 ml. of the test enzyme was added and the contents of the tube swirled at the beginning of the incubation period. The tubes were incubated for three hours, at the end of which time, 10 ml. of 0.6 N trichloroacetic acid were added. The contents were shaken vigorously and centrifuged at 1,000 r.p.m. for 12-15 minutes. Following this, 1.0 ml. of the supernatant was transferred to a 125 ml. Erlenmeyer, using a volumetric pipette, and 4.0 ml. of distilled water was run in from a burette. To each of the flasks, 10.0 ml. of 0.5 N NaOH were added, followed by a drop-wise addition of 3.0 ml. of Folin-Ciocalteu reagent diluted 1:2 with distilled water. Color development of tyrosine and tryptophan released during the proteolysis was allowed for five minutes. Intensity of color developed, representing the extent of enzyme activity, was determined with a Klett-Summerson photoelectric colorimeter, using Filter No. 60.

Blank determination

Casein substrate and the test enzyme had a blank reading. This value was obtained by adding the test enzyme to the casein substrate after addition of 10.0 ml. 0.6 N trichloroacetic acid. The color reading of the blank for each determination was subtracted from that of the corresponding test. The micrograms of tyrosine per 5.0 ml. substrate liberated due to enzyme activity then were read off from the standard curve for L-tyrosine.

Estimation of proteolytic activity with hemoglobin substrate

A solution of 3.0 per cent hemoglobin (Merck) was dialyzed in a cellophane tube for 24 hours against distilled water and centrifuged. The supernatant was adjusted to pH 5.0. The volume was made up to correspond to 2.5 per cent solution. Merthiolate in the concentration of 1.0 mg. per 40.0 ml. substrate was added. In making the determination, 0.2 ml. of the test enzyme was added to 5.0 ml. portions of this substrate in test tubes and incubated for three hours in a water bath at 37.5° C. At the end of the incubation period, 10.0 ml. of 0.3 N trichloroacetic acid were added and the tubes shaken and centrifuged at 1,000 r.p.m. for 12-15 minutes. To 1.0 ml. of the supernatant in an Erlenmeyer were added 4.0 ml. distilled water, 10.0 ml. 0.5 N NaOH and 3.0 ml. of 1:2 Folin and Ciocalteu

reagent. The color developed was read in the Klett-Summerson photoelectric colorimeter. Blank determinations for this substrate were made in the same manner as for casein. Readings of the blank were subtracted from the test determination and the micrograms of tyrosine liberated per 5.0 ml. of the substrate read off from the standard L-tyrosine curve.

Standard tyrosine curve and expression of enzyme activity

Various concentrations of L(-)tyrosine (Merck) dissolved in dilute alkali were taken in 5.0 ml. portions. Instead of 0.2 ml. test enzyme and 10.0 ml. portions of trichloroacetic acid used in actual determinations, 10.2 ml. of distilled water were added to each 5.0 ml. of standard tyrosine solution. Out of this, 1.0 ml. portions were transferred to a 125 ml. Erlenmeyer, followed by 4.0 ml. distilled water, 10.0 ml. 0.5 N NaOH and 3.0 ml. Folin and Ciocalteu reagent diluted 1:2. Color developed was read in a Klett-Summerson photoelectric colorimeter, using filter No. 60. The values obtained, corresponding to the micrograms of amino acid in 5.0 ml. of the original solutions, were plotted against colorimeter readings (Figure 1).

Exact amounts of tyrosine present in the standard solutions were determined by Kjeldhal-Gunning-Arnold method (1940).

Enzyme activity has been expressed as the micrograms

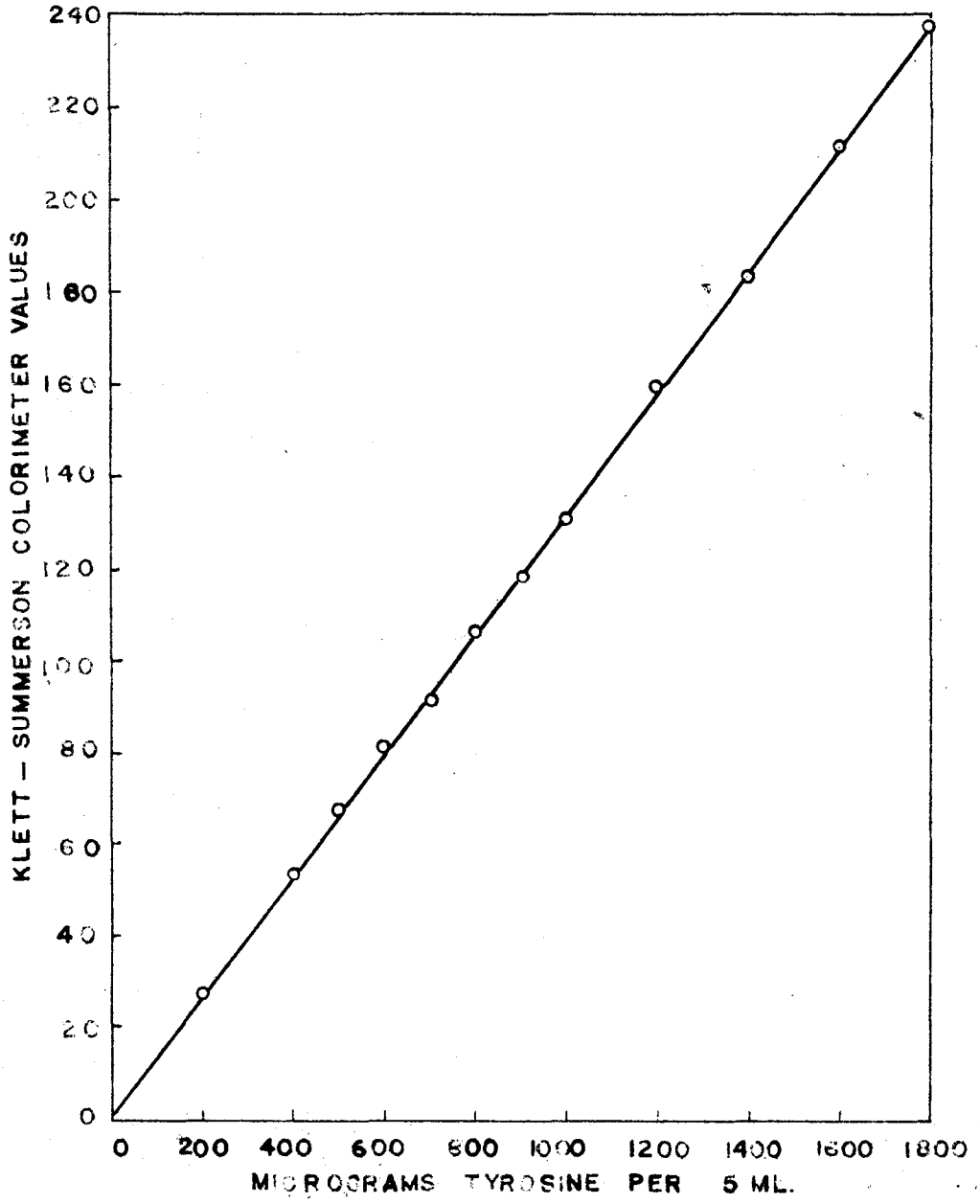

Figure 1. Standard curve relating micrograms of tyrosine to color produced by Folin-Ciocalteu reagent

of tyrosine liberated by 0.2 ml. of the test enzyme from 5.0 ml. substrate when incubated for three hours at 37.5° C.

Determination of Peptidase Activity

Preparation of the test enzyme

The procedure followed was the one employed by Berger et al. (1938a). A 24-hour culture of S. liquefaciens, culture C, transferred in V-8 broth for three days before use, was inoculated (1 per cent) into four liters of V-8 broth (0.5 per cent tryptone, 0.5 per cent tryptose, 0.5 per cent yeast extract, 1.0 per cent K_2HPO_4 and 20 per cent strained V-8 juice with pH adjusted to 7.2 and sterilized at 15 lb. for 15 minutes). The flasks were incubated at 35° C. for 24 hours and the reaction of the medium after growth was found to be pH 5.6. The cells were harvested using a Sharples super centrifuge run at 25,000 r.p.m. By this procedure, 10.0 g. of the wet cells were recovered and suspended in 15 ml. of distilled water. The pH of this material was adjusted to 6.8, using 1.0 N NaOH. The suspension was frozen and thawed for eight times in five days. After this treatment, the suspension was mixed with fine sea sand and ground in a mortar and pestle. Eighty ml. of distilled water were added and the mixture allowed to stand overnight at room temperature (ca. 72° F.) to permit

autolysis of the cells. A thin layer of toluene was added. The material was centrifuged in an angle centrifuge at 3,000 r.p.m. for 30 minutes. By this method, 80.0 ml. of the enzyme preparation were recovered. For convenience, this preparation henceforth will be referred to as the "original preparation". The final reaction of this preparation was pH 6.6.

Preparation of the substrates

Glycyl-L-leucine and DL-alanylglycine were the two dipeptides used as substrates for the study of the peptidases of the organism.

Glycyl-L-leucine was prepared in M/30 concentration in distilled water. Sodium citrate, potassium phosphate and sodium borate buffers, in the effective concentration of 0.01 M, were used in pH ranges 4.0-6.0, 6.1-8.0 and 8.1-10.0, respectively.

DL-alanylglycine was prepared in a similar way, except that M/15 concentration was used, since the compound used was a mixture of both the optically-active isomers. It is generally believed that D-forms are not attacked by biological systems.

Activity determination

In each determination, 1.0 ml. of the proper dilution

of the "original preparation" was added to 2.0 ml. of the buffered substrate. The contents were mixed and incubated in a water bath at 37.5° C. for periods which varied in different experiments. Both at the beginning and at the end of the incubation period, 1.0 ml. of the enzyme-substrate mixture was titrated. The procedure followed for titration was essentially that of Linderstrom-Lang (1927) as used by Berger et al. (1938a), except that in place of N/20 alcoholic HCl, N/50 alcoholic HCl was used for titration.

The indicator used was 4-benzene-azo-1-napthylamine (napthyl red), 0.1 g. being dissolved in 100 ml. of 95 per cent alcohol. For each titration, two drops of the indicator were used. A 15 ml. quantity of acetone (C.P.) was used for each determination.

Immediately after the addition of the test enzyme to the substrate, 1.0 ml. was withdrawn and transferred to a 50 ml. Erlenmeyer flask, followed by the addition of two drops of the indicator. A standard solution of N/50 HCl in 90 per cent alcohol was run in from a microburette until the contents in the flask turned distinctly reddish in color. At this stage, a part of 15 ml. of acetone was added, thus changing the color of the indicator to yellow. More alcoholic HCl was added to bring the color of the mixture to orange-yellow. At this point remainder of acetone was added and the titration continued to the

orange color of the end-point. Per cent hydrolysis was calculated after subtracting the initial titration value from the value obtained for titration at the end of the incubation period.

The results are expressed as per cent hydrolysis of one isomer. In case of the DL- form, per cent of one linkage of one optical component (L-) was calculated.

Procedure for activation

In case of activation trials using various compounds for the purpose, the desired amount of the "original preparation" was made up with distilled water to 0.5 ml. instead of 1.0 ml., as was the case for activity determination procedure outlined above. To each 0.5 ml. portion of this enzyme taken in a test tube, 0.5 ml. quantities of $2x10^{-2}$, $2x10^{-3}$, $2x10^{-4}$ and $2x10^{-5}$ M solutions of different test-compounds were added, in order to bring their effective concentration to $1x10^{-2}$, $1x10^{-3}$, $1x10^{-4}$ and $1x10^{-5}$ M, respectively. The enzyme-activator mixtures were incubated in a water bath at 37.5° C. for 45 minutes. At the end of this period, 2.0 ml. of the substrate at previously adjusted pH levels were added. Aliquot samples (1.0 ml.) were tested for activity, both at the beginning and the end of the incubation interval.

Determination of Coagulating Activity

The milk substrate for the coagulation test was made up by dissolving 10.0 g. of skim milk powder in 100 ml. of distilled water and heated in the Arnold steamer for 7-8 minutes. This reconstituted milk was cooled to about 20° C. The requisite amount of 5.0 per cent lactic acid was added to the milk drop by drop, while shaking, to bring the pH to 5.58-5.60. The adjusted milk was allowed to equilibrate for one hour. This pH level for milk was chosen arbitrarily, since trials made failed to indicate an optimum pH for coagulating activity (Table 13).

For each determination, 5.0 ml. portions of the milk substrate were taken in test tubes and placed in a water bath at 37.5° C. and the contents allowed to come to this temperature, when 0.2 ml. portions of the test enzyme (the same preparation as used for the proteinase determinations) was added to the tubes and mixed by gentle swirling. Coagulation was observed by twirling the tubes and the first sign of flakes sticking on the sides of the tubes was considered the point of coagulation. The coagulation time was taken as the least time required for 0.2 ml. of the test-material to curdle 5.0 ml. of milk substrate, when incubated at 37.5° C.

Counting of Viable Bacteria in Growth Media

The plating procedures employed were exactly the same as embodied in the ninth edition of "Standard Methods for the Examination of Dairy Products" (1948). Incubation temperature of 35° C. was used throughout this work.

pH Determinations

The Leeds and Northrup glass electrode potentiometer was used throughout most of the study for adjusting pH in growth media or for the substrates used. However, towards the later part of this work a Beckman glass electrode set was used. The quinhydrone-calomel potentiometer (Leeds and Northrup) was employed for final pH determinations in small quantities of growth media.

EXPERIMENTAL RESULTS

The observation that S. liquefaciens produces a proteolytic enzyme and a rennet-like, coagulating enzyme has been made by several workers and has been accepted as a characteristic of this organism (Breed et al., 1948). However, in all of these reports, milk is the only medium used for the growth of the organism. Gorini (1942) observed that the proteinases and peptidases of the "acido-proteolytes" of milk are not stable in acid pH and are destroyed below pH 4.5. No attempt seems to have been made to study this enzyme system by growing the organism in a suitable medium that would hold the pH in a range favorable for the stability of the proteolytic and coagulating enzymes. There is no reference in the literature concerning the stability of the so called rennin-like enzyme produced by this organism.

The purpose of this section will be to report on the attempts made at evolving a suitable growth medium for S. liquefaciens that would permit maximum production of both the proteolytic and coagulating enzymes. The main purpose in deriving such a medium was to facilitate the isolation of these two enzymes and, if possible, to separate the proteolytic enzyme fraction from the coagulating one.

However, these latter topics will be dealt with in the subsequent sections of this thesis.

Deriving a Suitable Medium for Production of Proteolytic and Coagulating Enzymes

Culture N and C were grown in a broth containing 0.5 per cent peptone, 0.3 per cent beef extract and 0.5 per cent lactose, at pH 6.7. Both these organisms grew quite well in this broth, as judged by the turbidity and drop in pH of the growth medium. However, no coagulating activity could be detected in the centrifuged growth medium as determined by the milk coagulation test, although these organisms did exhibit coagulating activity when grown in milk under similar conditions. The pH of the two media after growth was quite comparable.

Because of the possibility that the absence of enzyme production might be due to lack of calcium and magnesium ions in the medium, as was found by Merrill and Clark (1928) in case of gelatinase production by Ps. putrefaciens, 10 mg. $MgSO_4$ and 4 mg. $CaCO_3$ were added per 100 ml. of the above broth. As shown by Table 1, use of this modification did not result in any enzyme production.

At this stage it was thought advantageous to grow the organisms on the simplified amino acid medium as worked out by Niven and Sherman (1944). The medium was prepared

Table 1. Effect of Adding Calcium and Magnesium to Nutrient Broth on the Production of Coagulating Enzyme by Cultures N and C

Growth medium	Culture N		Culture C	
	Final pH	Coagulation time	Final pH	Coagulation time
		min.		min.
Broth, pH 6.70	4.60	>60	4.68	>60
Broth plus Ca and Mg, pH 6.70	4.66	>60	4.80	>60
Litmus milk, pH 6.70	4.52	16	4.81	18

entirely as outlined by these workers. N and C strains were inoculated in this medium. While strain N grew quite well on 24-hour incubation, culture C failed to grow within the same period. Addition of thiamine in the concentration of 5 γ per 5 ml. of the medium resulted in growth of culture C.

In order to make the growth conditions uniform, both N and C were grown for 24 hours on Niven and Sherman's medium with thiamine added to it before sterilizing. The test enzyme failed to coagulate milk within 60 minutes. It might be pointed out that the pH of the medium after growth of both N and C was found to be below pH 4.5, a reaction at which destruction of the enzyme occurs, as reported by Gorini (1942).

As a next step, attempts were made to modify the growth medium to prevent the drop in pH to a point where the enzymes might be inactivated. This was sought to be accomplished first, by increasing the buffering capacity of the medium and, second, by decreasing, where possible, the amount of fermentable sugar in the medium. Addition of 1 per cent KH_2PO_4 and 0.2 per cent lactose seemed to satisfy these conditions.

Niven and Sherman's simplified amino acid medium therefore was modified by replacing 0.5 per cent glucose with 0.2 per cent lactose and by including 1.0 per cent KH_2PO_4 in place of 0.5 per cent K_2HPO_4. Thiamine, in the concentration of 5γ per 5 ml. also was included in this medium, since on preliminary trials, two (C and B) of the nine strains failed to grow without added thiamine. The medium was tubed in 5 ml. quantities and sterilized for 13 minutes at 15 lb. pressure. All of the nine cultures were grown for 24 hours in this modified Niven and Sherman's medium. The cultures were centrifuged to remove the cells and the supernatant tested for proteolytic and coagulating activities. Plate counts were made before centrifuging the medium. Representative results of these determinations are embodied in Table 2.

All these nine cultures also were grown for 18 hours in litmus milk. Representative results of these tests are given in Table 3.

Table 2. Production of Coagulating and Proteolytic Enzyme Activities by Different Strains Grown on Modified Niven and Sherman's Amino Acid Medium (pH 6.9) for 24 Hours at 35° C.

Culture	Final pH	Coagulation time min.	Proteo-* lysis	Plate count ($x10^7$/ml.)
20	6.01	>60	8	185
0	6.16	>60	0	175
Y	6.13	>60	0	150
19	6.08	>60	8	188
7	6.10	>60	8	167
E	6.20	>60	8	167
B	6.31	>60	8	93
N	6.02	>60	15	172
C	6.02	>60	8	186

*Expressed as micrograms of tyrosine/5 ml. of reaction medium; proteolysis measured on casein substrate, pH 7.4.

Table 3. Production of Coagulating and Proteolytic Enzyme Activities by Different Strains Grown on Litmus Milk for 18 Hours

Culture	Final pH	Coagulation time (min.)	Proteolysis*	Plate count ($x10^7$/ml.)
20	4.56	14	308	291
O	4.61	11	338	251
Y	4.71	17	270	183
19	4.69	16	225	240
7	4.75	10	338	214
E	4.78	19	210	218
B	4.94	16	255	174
N	4.75	11	353	256
C	4.88	10	300	284

*Expressed as micrograms of tyrosine/5 ml. of reaction medium; proteolysis measured on casein substrate, pH 7.4.

It will be seen that all these cultures grew satisfactorily in both these growth media. However, no proteolytic or coagulating activities could be detected for any of the test organisms in the modified Niven and Sherman's medium, although pH after growth stayed well above pH 4.5. In litmus milk, all the strains produced both proteolytic and coagulating enzymes, although the activity levels differed with the individual strains. A marked parallelism between coagulating and proteolytic activities was observed.

The previous trials seemed to indicate that pH probably was not the only factor involved. There apparently was something else present in milk which permitted enzyme production even though the final pH dropped quite low. In order to get more information on this point, milk solids in the growth medium were increased from 10 to 20 and 30 per cent. Culture N was grown for varying lengths of time in milk preparations of various concentrations. The supernatant test material was examined for pH, coagulating ability and proteinase activity, using for the latter the method of Anson (1938), employing 2.5 per cent hemoglobin substrate at pH 5.0. It might be mentioned that this particular method was used in the earlier stages of this investigation until more information became available. Later, casein at pH 7.4, the optimum for proteolysis, was adopted.

Results obtained using the hemoglobin procedure are presented here only for use on a comparative basis.

Representative data are given in Table 4. From these results, it appears that the increase in milk solids resulted in a considerable increase in both the proteolytic

Table 4. Effect of Increased Milk Solids in Litmus Milk on Coagulating and Proteolytic Activities of Culture N

Milk solids conc.	Value measured	Age of cultures (hr.) when tested: 12	18	24	36	48
%						
10	Final pH	5.15	4.76	4.50	4.50	4.60
	Coagulation*	9	8	12	>60	>60
	Proteolysis**	405	465	435	53	8
20	Final pH	5.20	4.78	4.58	4.55	4.60
	Coagulation*	8	8	5	6	8
	Proteolysis**	495	525	555	893	705
30	Final pH	5.19	4.80	4.55	4.58	4.59
	Coagulation*	10	8	5	5	7
	Proteolysis**	503	518	533	998	938

*Time in minutes

**As micrograms of tyrosine/5 ml. of reaction medium; proteolysis measured on hemoglobin substrate, pH 5.0.

and coagulating activities. In case of 10 per cent milk, there was very little activity on 36 hours incubation, while at 20 and 30 per cent milk solids there was considerable

proteolytic and coagulating activity up to 48 hours of incubation. This would seem to be due to the increased concentration of protein in the medium in the latter case.

In order to determine the effect on enzyme production of addition of different peptones to milk used for growth of the organisms, Bacto tryptone, Bacto tryptose, Bacto proteosepeptone No. 3 and Bacto peptonized milk in the concentration of 1.5 per cent were added individually to litmus milk containing 10 per cent milk solids. Representative results of two trials are given in Table 5. In these runs hemoglobin was used as a substrate for determination of proteolytic activity; hence the results should be treated only in a comparative way. There was marked reduction in both proteolytic and coagulating activities when any of the five additional ingredients was included in milk, as compared to the control without added peptone.

The evidence thus far collected pointed to the possibility that the production of both the proteolytic and the coagulating enzymes required whole protein in the growth medium. In addition to this, addition of peptones seemed to have a retarding effect on the production of the two activities. Therefore, attention was directed toward developing a suitable medium containing casein or possibly some other protein, with enough buffering capacity in the medium, a suitable amount of fermentable carbohydrate and

Table 5. Influence of Addition of Different Peptones to Milk on Coagulating and Proteolytic Activities of Culture N

Addition to growth medium	Value measured	Age of cultures (hr.)			
		12	18	36	48
1.5% Tryptone	Final pH	5.18	4.85	4.70	4.63
	Coagulation*	26	30	43	>60
	Proteolysis**	225	188	105	45
1.5% Tryptose	Final pH	5.13	4.79	4.60	4.60
	Coagulation*	33	41	58	>60
	Proteolysis**	128	98	98	0
1.5% Proteose-peptone	Final pH	5.19	4.75	4.65	4.58
	Coagulation*	48	56	>60	60
	Proteolysis**	105	113	15	0
1.5% Peptonized milk	Final pH	5.15	4.80	4.59	4.53
	Coagulation*	50	58	>60	>60
	Proteolysis**	113	105	8	8
Litmus milk (control)	Final pH	5.20	4.78	4.55	4.50
	Coagulation*	8	8	15	>60
	Proteolysis**	398	465	360	15

*See footnote on Table 4

**See footnote on Table 4

any other growth factors needed by these organisms, that would permit both good growth and high enzyme production.

After preliminary trials (Table 9) it was found that the vitamin-test casein medium as described under Experimental Methods would prove quite satisfactory. In order

to decide if inclusion of inorganic salts was necessary in such a medium, cultures N and C were grown on vitamin-test casein medium with and without inorganic salts for 24 hours and tested for final pH and proteolytic and coagulating activites, as well as for bacterial numbers. Representative results of these trials are given in Table 6. It will be seen from the results obtained that addition of inorganic salts to this medium, at the levels used, did not result in either better growth or increased proteolytic and coagulating activities. Therefore, inorganic salts were not included in the medium in subsequent trials.

Table 6. Effect of Addition of Inorganic Salts to Vitamin-Test Casein Medium on Coagulating and Proteolytic Enzyme Activities Produced by Strains N and C

Value measured	Culture N		Culture C	
	Without[1] salts	With[2] salts	Without[1] salts	With[2] salts
Final pH	5.82	5.78	5.83	5.79
Coagulation**	6	7	22	24
Proteolysis*	570	533	90	98
Plate count ($x10^7$/ml.)	250	260	240	270

[1]Initial pH 6.40

[2]50 mg. NaCl, 10 mg. $MgSO_4$, 5 mg. $FeCl_3$, 10 mg. $MnSO_4$ and 10 mg. $CaCO_3$ added/100 ml. of basal medium, initial pH 6.4

*See footnote on Table 2 **Time in minutes

The effect of omission of individual vitamins from the vitamin-test casein medium on the growth and the production of proteolytic and coagulating enzyme activities was investigated. Representative data for cultures N and C are presented in Tables 7 and 8. Identical results were obtained for both the strains, except that in the complete medium, culture C produced much smaller quantities of both the enzymes as compared to its production of either of the enzymes in litmus milk (Table 3). Individual omission of riboflavin, calcium pantothenate and nicotinic acid resulted in very feeble growth and absence of a detectable level of either type of enzyme activity in cases of both N and C. Complete omission of all of the six vitamins had a similar effect on both. Individual omission of pyridoxine, biotin and folic acid had some retarding effect on growth, as determined by the plate count and drop in the pH of the growth medium, and for enzyme production for both strains. Culture C produced much less enzyme activity, even though the counts for both were much the same.

All the nine strains subsequently were tested on this vitamin-test casein medium; representative data are given in Table 9. It might be pointed out that in case of culture B, 5.0 γ thiamine per 5.0 ml. was included in this medium, since the organism failed to grow satisfactorily without this addition. There were individual variations for the two

Table 7. Effect of Omission of Vitamins from Vitamin-Test Casein Medium (pH 6.60) on the Coagulating and Proteolytic Enzyme Activities Produced by Strain N

Vitamin omitted	Final pH	Coagulation time (min.)	Proteo-lysis*	Plate count ($x10^7$/ml.)
All	6.43	>60	8	6
Riboflavin	6.42	>60	0	56
Calcium pantothenate	6.42	>60	0	15
Nicotinic acid	6.38	>60	0	30
Pyridoxine	6.08	13	323	190
Biotin	6.11	15	285	197
Folic acid	6.05	12	345	189
None	5.96	8	510	246

*See footnote on Table 2

Table 8. Effect of Omission of Vitamins from Vitamin-Test Casein Medium (pH 6.60) on the Coagulating and Proteolytic Enzyme Activities Produced by Strain C

Vitamin omitted	Final pH	Coagulation time (min.)	Proteo-lysis*	Plate count ($x10^7$/ml.)
All	6.51	>60	0	4
Riboflavin	6.36	>60	0	21
Calcium pantothenate	6.48	>60	0	6
Nicotinic acid	6.40	>60	15	61
Pyridoxine	6.27	46	38	177
Biotin	6.33	51	38	123
Folic acid	6.30	38	30	173
None	6.00	26	83	226

*See footnote on Table 2

Table 9. Production of Coagulating and Proteolytic Enzyme Activities by Different Strains on Vitamin-Test Casein Medium (pH 6.92)

Culture	Final pH	Coagulation time (min.)	Proteo-lysis*	Plate count ($x10^7$/ml.)
20	6.32	9	480	74
O	6.31	7	623	182
Y	6.37	15	315	153
19	6.34	8	555	100
7	6.34	12	408	157
E	6.38	25	188	172
B[1]	6.39	28	150	199
N	6.29	7	608	221
C	6.38	29	68	222

*See footnote on Table 2

[1]Contained 5.0 γ thiamine/5 ml. in addition to basal medium

enzyme activities for the various strains examined, as was found to be the case when litmus milk was used as the growth medium (Table 3).

The fact that culture C failed to produce adequate amounts of the two enzymes in this medium has already been pointed out. The possibility that inclusion of certain other nutrients might increase the enzyme production to at least about the same level as is produced by this strain in milk therefore was investigated. Vitamin B_{12} in the concentration of 100 mγ per 5.0 ml. was added to the vitamin-test casein medium before sterilizing and both cultures N and C were grown in the supplemented medium for 24 hours. The supernatant was tested as usual for proteolytic and coagulating activities. No noticeable effect on culture N was detected but there was a three-fold increase in both proteolytic and coagulating activities in the case of strain C, as compared to the enzyme produced on this same medium without any added vitamin B_{12}. Addition of this vitamin did not result in any increase in the bacterial numbers as determined by the plating procedure. Later on, 2.0 mγ of B_{12} per 5.0 ml. was found to be adequate, so the quantities of this nutrient added were reduced in subsequent trials. All of the nine strains were grown on vitamin-test casein medium with B_{12} added in the concentration of 2.0 mγ per 5.0 ml. Representative results are presented in Table 10.

Table 10. Production of Coagulating and Proteolytic Enzyme Activities by Various Strains on Vitamin-Test Casein Medium Supplemented with 2.0 mγ Vitamin B_{12}/5 ml. (pH 6.92)

Culture	Final pH	Coagulation time (min.)	Proteo-lysis*	Plate count ($x10^7$/ml.)
20	6.32	9	473	88
O	6.33	7	615	189
Y	6.34	16	330	147
19	6.33	9	525	95
7	6.34	13	383	167
E	6.35	23	218	160
B[1]	6.36	26	195	192
N	6.30	6	645	189
C	6.32	10	420	195

*See footnote on Table 2

[1]Contained 5.0γ thiamine/5 ml. in addition to basal medium

Except in the case of culture C, no detectable difference was found in either the proteolytic or the coagulating activities or in the total plate count.

Since vitamin B_{12} is known to contain about 4 per cent cobalt (Brink et al., 1949), it was suspected that the

increased enzyme activity might be due to the presence of this metal ion in vitamin B_{12}. $CoSO_4.7H_2O$ in concentrations of 5, 15 and 100 mγ per 5.0 ml. was included in the vitamin-test casein medium. Addition of cobalt in these concentrations failed to duplicate the effect produced by vitamin B_{12} in case of strain C. In order to verify that B_{12} was not merely acting as an activator for the enzyme system, culture C was grown in the vitamin-test casein medium without any added B_{12} and the supernatant test enzyme was incubated with B_{12} in the concentration of 2.0 and 20.0 mγ per 5 ml., for 30 minutes at room temperature. This material was tested for both proteolytic and coagulating activities in the usual way. No increase in either of these activities over the control could be detected.

In order to see if comparatively poor production of both the enzymes in vitamin-test casein medium could be increased to any extent, several nutrients were included in this basal medium. Addition of thiamine, Tween 80, sodium acetate, ascorbic acid and para-aminobenzoic acid had no effect on the production of the coagulating enzyme by the strains C, B and E (Table 11).

Effect on enzyme production of addition of gelatin and casein to peptone broth

The information thus far obtained seemed to indicate

Table 11. Effect of Addition of Nutrients to Basal Vitamin-Test Casein Medium on the Coagulating Activity of Cultures C, B and E

Nutrient added/5 ml.	Coagulation time (min.) using culture:		
	C	B	E
Thiamine, 5 γ	30	31	30
Thiamine, 5γ; B_{12}, 20 mγ	10	28	29
Tween 80, 10 mg.; sodium acetate, 10 mg.	28	*	27
Tween 80, 10 mg.; sodium acetate, 10 mg.; B_{12}, 2mγ	10	*	29
Ascorbic acid, 2.5 γ	33	*	32
p-Aminobenzoic acid, 2.5γ	31	*	32
Ascorbic acid, 2.5γ; p-Aminobenzoic acid, 2.5γ; Inositol, 5γ	32	*	29
None	29	*	27

*No growth obtained on 24-hour incubation

the adaptive character of the proteolytic and coagulating enzyme activities produced by these organisms. In order to further elucidate this point, four of the strains, N, C, B and Y were grown in the following three different peptone broths, with pH adjusted to 6.9: (1) Peptone broth, containing 0.5 per cent Bacto peptone, 0.3 per cent beef

extract, 0.2 per cent lactose and 1.0 per cent K_2HPO_4; (2) Peptone broth 1, with 2.0 per cent gelatin added; and, (3) Peptone broth 1, with 2.0 per cent technical casein (Fischer Scientific) added. In case of all the four strains (Table 12), there was production of both the enzyme fractions in any of the three media. However, maximum enzyme production occurred in the gelatin peptone broth and the least in the peptone broth, with that in the casein peptone broth occupying an intermediate place.

Table 12. Effect of Addition of Gelatin and Casein to Peptone Broth on the Production of Coagulating and Proteolytic Enzyme Activities

Medium	Culture	Final pH	Coagulation time (min.)	Proteolysis*	Plate count ($x10^7$/ml.)
Peptone broth (pH 6.90)	Y	6.03	31	173	153
	B	6.16	35	135	142
	N	6.05	24	233	170
	C	6.12	29	113	180
Peptone broth with gelatin (pH 6.90)	Y	5.99	16	308	169
	B	6.09	28	180	150
	N	5.94	10	435	187
	C	5.94	14	300	211
Peptone broth with casein (pH 6.90)	Y	6.08	17	300	181
	B	6.18	29	165	173
	N	5.95	13	375	195
	C	6.05	18	240	196

*See footnote on Table 2

Study of Some of the Characteristics of the Extracellular Proteolytic and Coagulating Enzyme Activities

Determination of optimum pH for proteolytic activity

In order to find the pH of optimum activity for the extracellular proteolytic activity, culture C was grown on the casein medium (see Experimental Methods) for 24 hours and centrifuged to remove the cells. The procedure for proteinase determination used was the same as described under Experimental Methods, except that sodium citrate and sodium borate in the effective concentration of 0.05 M were used in place of potassium phosphate in the pH ranges of 4.0 to 6.0 and 8.1 to 10.0, respectively. In the pH range of 6.1 to 8.0, KH_2PO_4 was used as usual. For each 5.0 ml. portion of casein substrate adjusted to various pH levels, 0.2 ml. of the test enzyme was added. Representative data are plotted in Figure 2.

An identical experiment was run, using 1.0 per cent lactalbumin (Supplee Research Corp.), in place of casein substrate. Representative data also are presented in Figure 2.

It will be observed that optimum pH for both casein and lactalbumin lies in the neighborhood of pH 7.4 to 7.5. There is a sharp decline in the activity of the enzyme on either side of the optimum.

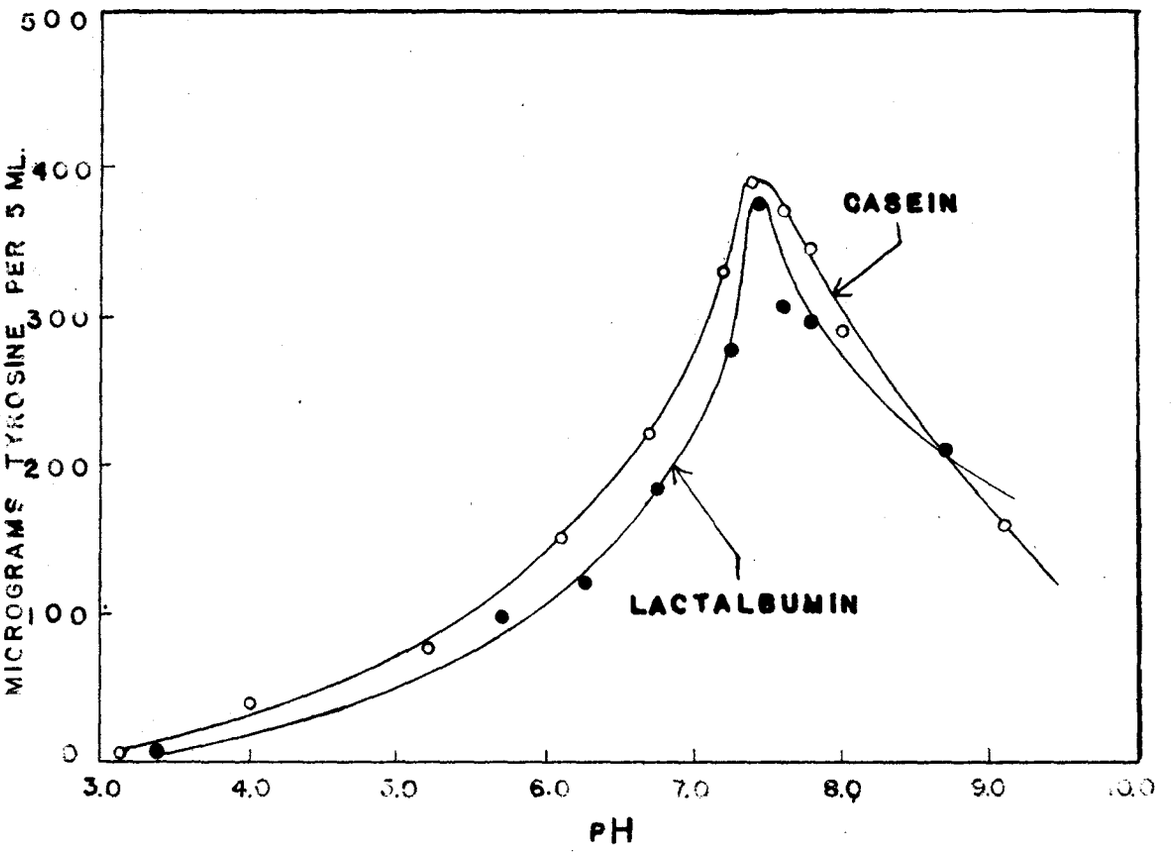

Figure 2. pH optima for proteinase action against casein and lactalbumin (0.2 ml. test enzyme per 5 ml. substrate incubated for 5 hours at 37.5° C. in both cases)

Effect of varying concentration of enzyme on casein digestion

Relationship of enzyme concentration to proteolysis of the casein substrate was studied. Different quantities of test enzyme (supernatant from 18 hour litmus milk culture of strain C, final pH 4.90) varying from 0.1 to 1.0 ml. were added to 5.0 ml. of the substrate. Contents of each tube were made up to 6.0 ml. by the addition of distilled water. The enzyme-substrate mixtures were incubated for three hours at 37.5° C. The rest of the procedure adopted was the same as has been used throughout this study. Milliliters of enzyme plotted against micrograms tyrosine released per 5.0 ml. casein substrate are presented in Figure 3. There seems to be a direct relationship between enzyme concentration and the protein breakdown up to 0.4 ml. quantity of the enzyme, with a subsequent curving off. Tyrosine values up to 500 micrograms fall on the linear portion of the curve, with increasing deviations from linear as the values increase above 500 micrograms.

Effect of time of incubation on digestion of casein

In this case, 70.0 ml. of 1.0 per cent casein substrate were taken in a flask and allowed to equilibrate in a water bath at 37.5° C. To this was added 2.8 ml. of the test enzyme (supernatant from 18 hour litmus milk culture of strain C) followed by gentle mixing. Immediately after the

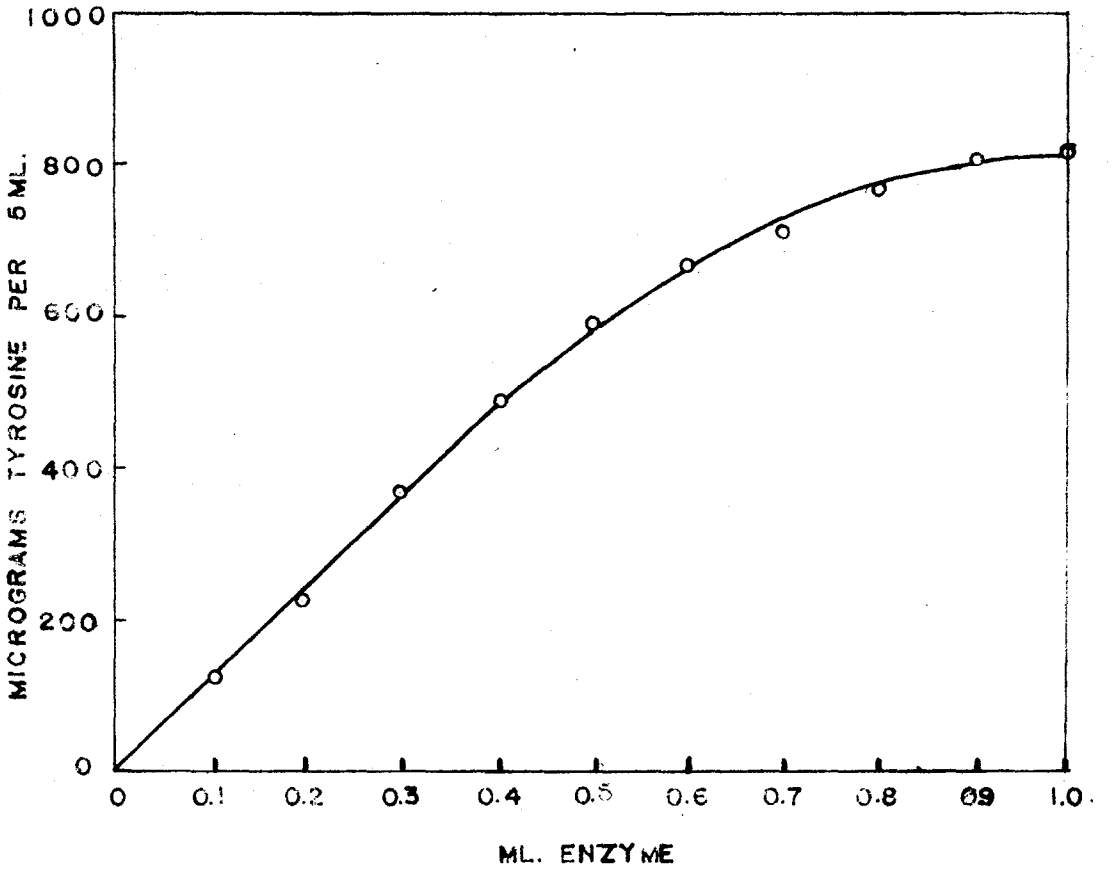

Figure 5. Effect of enzyme concentration on casein digestion (ml. test enzyme per 6 ml. casein substrate pH 7.4, incubated for 3 hours at 37.5° C.)

addition of the test enzyme to the substrate, 5.0 ml. of the mixture were transferred to a tube containing 10.0 ml. of 0.6 N trichloroacetic acid. This first tube corresponded to the zero time of enzyme activity. To the rest of the eleven empty tubes which had been allowed to stand in the water bath, 5.0 ml. aliquot samples from the enzyme-substrate mixture were transferred and incubated for different intervals of time. At the end of each interval, 10.0 ml. of 0.6 N trichloroacetic acid were added to each of the tubes in order to stop the reaction. The rest of the determination for proteolysis was made as usual. It will be seen (Figure 4) that up to 4.5 hours of incubation, there is a direct relationship between the time of incubation and breakdown of casein.

Determination of optimum pH for coagulating activity

Steamed skim milk (10.0 per cent milk powder) was adjusted to different pH levels by addition of 5.0 per cent lactic acid. The adjusted milk (5.0 ml. portions) was taken in tubes and placed in a water bath at 37.5° C. Test enzyme (0.2 ml. of the supernatant from 18 hour litmus milk culture of strain C) was added to each tube and the time of coagulation noted. The results (Table 13) show that if milk pH is"plotted"against time of coagulation, a direct relationship between the two values is demonstrated.

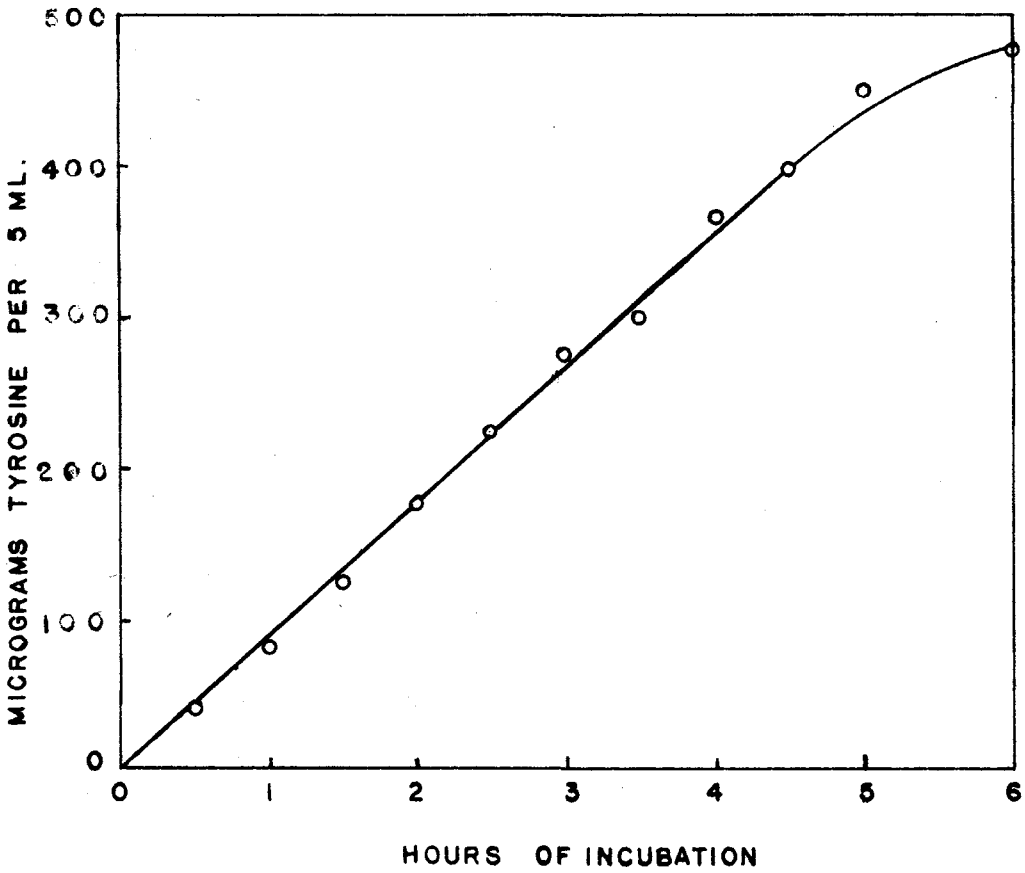

Figure 4. Effect of time of incubation on casein digestion (0.2 ml. test enzyme per 5 ml. casein substrate, pH 7.4, at 37.5° C.).

Table 13. Effect of pH of Milk Substrate on Coagulating Activity of Supernatant from 18 Hour Litmus Milk Culture of C

pH of milk substrate	Coagulation time (min.)
5.48	7
5.60	12
5.72	18
5.82	24.5
5.95	31
6.06	37
6.19	42
6.32	48
6.52	61
6.70	70

Effect of enzyme concentration on coagulating activity

Varying amounts of test enzyme (supernatant from 18 hour litmus milk culture of strain C) were added to 5.0 ml. of milk substrate adjusted to pH 5.75. In each case the volume of the enzyme substrate was made up to 6.0 ml. and the time of coagulation noted. The data presented in Table 14 show that there is a fairly good relationship

Table 14. Effect of Enzyme Concentration on Milk Coagulation (Test-enzyme: Supernatant from 18 hour litmus milk culture of C; pH of milk substrate: 5.75)

Ml. enzyme/6 ml.	Coagulation time	(ml. x time)
	min.	
0.1	44	4.40
0.2	22.5	4.50
0.3	14.5	4.35
0.4	10.5	4.20
0.5	8	4.00
0.6	6.75	4.05
0.7	5.5	3.85
0.8	5	4.00
0.9	4.5	4.05
1.0	4.25	4.25

between the enzyme activity and coagulation time up to 0.4 ml. quantities of the enzyme; that is, when the coagulation time is 10 minutes or greater. Below this figure there appears to be slight tendency for the coagulation time to be slightly shorter than would be calculated. The error in accurate reading of the tests when shorter times are used,

as well as the probable greater ease of detecting flocs when the enzyme concentration is higher, may account for some of the slight deviation.

Effect of heat on the proteolytic activity of the enzyme

Strain C was grown for 18 hours on litmus milk, the buffering capacity of which had been increased by the addition of 5 ml. of separately sterilized 20 per cent solution of K_2HPO_4. The supernatant (pH 5.0) was adjusted to different pH levels by a very gradual addition of 1.0 N NaOH. For each heat treatment trial, 2.0 ml. of the test enzyme at each pH were placed in a test tube and heated at the desired temperature for the various lengths of time, after the contents had reached that particular temperature. After the heat treatment, the tubes immediately were plunged in cold water (ca. 15° C.) to cool the contents. From each heat-treated sample of the test enzyme, 0.2 ml. portions were tested for proteolytic activity on casein substrate as usual. Results of representative trials are given in Table 15.

Maximum stability of the enzyme at all three temperatures used seemed to occur at pH 6.0 to 8.0, with little variation over this range. The enzyme was inactivated quite rapidly at pH 5.0. At pH 9.0, while the enzyme was quite stable at 50 and 55° C., it was much less stable when

Table 15. Effect of Heat at Different pH Levels on the Proteolytic Activity of Supernatant from 18 Hour Litmus Milk Culture of C

pH of test enzyme	Proteolytic activity* after heating at (°C.) for (min.):								
	Unheated enzyme	50			55			61.7	
		5	15	25	5	10	15	2	5	10
5.0	300	225	173	135	143	75	8	0	0	0
6.0	308	255	210	173	158	98	60	75	23	0
7.0	293	233	203	180	173	113	68	83	30	0
8.0	285	233	195	165	165	105	68	68	38	0
9.0	263	218	173	135	150	105	45	60	0	0

*See footnote on Table 2

heated at 61.7° C. The data show that even in the pH range of greatest stability the enzyme was inactivated completely by normal pasteurizing temperatures.

Effect of heat on the coagulating activity of the enzyme

The same test enzyme, heat-treated at different temperatures, as used for heat-inactivation of proteolytic activity was tested for corresponding coagulating activity by the usual procedure. Representative data are presented in Table 16. Here again, the enzyme seemed to have its maximum

Table 16. Effect of Heat at Different pH Levels on the Coagulating Activity of Supernatant from 18 Hour Litmus Milk Culture of C

pH of test enzyme	Coagulating activity* after heating at (°C.) for (min.):								
	Unheated enzyme	50			55			61.7	
		5	15	25	5	10	15	2	5	10
5.0	10	13	20	28	23	42	>60	>60	>60	>60
6.0	10	12	17	22	23	30	48	44	>60	>60
7.0	11	13	17	22	20	29	42	43	>60	>60
8.0	12	14	18	24	25	27	40	44	>60	>60
9.0	13	16	20	25	23	28	>60	52	>60	>60

stability in the pH range of 6.0 to 8.0. The test enzyme was much less resistant to heat at pH 5.0, while pH 9.0 was not as favorable for its stability as pH range of 6.0 to 8.0. However, at pH 9.0 there was less destruction of the enzyme as compared to that at pH 5.0. The coagulating activity was destroyed by the usual heat treatment employed in commercial pasteurizing.

Stability of the proteolytic activity at different pH levels when held at 2.2 and 30° C.

The test enzyme was prepared in the same way as was

done for the study of heat inactivation of the enzyme, as reported earlier in this thesis. Culture C was used in this case also. Initial activity of the enzyme adjusted to different pH levels was tested within 15 minutes after pH adjustment was completed. Merthiolate, in the concentration of 1.0 mg. per 40.0 ml., was added to the test-material to prevent any bacterial growth. The effect of different pH levels during a storage period of eight days is reported in Table 17.

There was considerable destruction of the test enzyme in the process of adjustment to pH levels of 4.0 and 4.5 and a slight inactivation at pH 9.0. At both 2.2 and 30° C., there was complete destruction of the proteolytic activity within 24 hours at pH 4.0 and 4.5. Both activities appear to be stable within the pH range of 5.3 to 9.0, when held at 2.2° C. up to eight days. At 30° C., there was more destruction at pH 5.3 and pH 9.0 after eight days, while the test enzyme was essentially unaffected between pH 6.4 and 8.5.

Stability of coagulating activity at different pH levels when held at 2.2 and 30° C.

The results of corresponding stability of the coagulating activity of the test enzyme held at 2.2 and 30° C. are also presented in Table 17. The stability of the coagulating activity corresponds very strikingly with that of the proteolytic activity of this test enzyme.

Table 17. Stability of Proteolytic and Coagulating Enzyme Activities at Different pH Levels when Held at 2.2 and 30° C. for Various Lengths of Time

pH of test enzyme**	Proteolysis* after:				Coagulation time (min.) after:			
	0 hr.	2 hr.	24 hr.	8 days	0 hr.	2 hr.	24 hr.	8 days
				2.2° C.				
4.0	98	15	0	-	35	>60	>60	--
4.5	150	68	0	-	22	45	>60	--
5.3	270	263	270	248	11	11	12	15
6.4	278	263	270	263	11	12	11	12
7.6	263	270	248	255	12	12	13	12
8.5	248	240	225	240	13	13	14	13
9.0	218	203	210	195	14	15	15	16
				30° C.				
4.0	98	0	8	-	35	>60	>60	--
4.5	150	45	8	0	22	>60	>60	>60
5.3	270	278	225	143	11	12	15	22
6.4	278	285	263	240	11	12	12	13
7.6	263	255	240	248	12	13	12	11
8.5	248	255	255	225	13	14	13	14
9.0	218	203	173	150	14	15	17	19

*See footnote on Table 2

**Supernatant from culture C grown 18 hours on litmus milk fortified with 1% K_2HPO_4 (pH 5.02)

Effect of addition of sodium oxalate to milk on the coagulating activity of the enzyme

It was noticed early during this investigation that longer steaming of reconstituted, low-heat skim milk powder used as milk substrate for milk coagulation test resulted in increased time of coagulation. In order to ascertain if this effect was due to the removal of calcium from milk due to longer heating, an experiment was run by adding different amounts of 1.0 per cent sodium oxalate to raw milk. This milk was heated in a water bath at 80° C. for 15 minutes, after the contents had reached that temperature. Control was run by heating the raw milk in a similar manner, without any addition of sodium oxalate. The results (Table 18) show that addition of 0.2 ml. of the sodium oxalate solution to 5.0 ml. of milk prevented coagulation by 0.4 ml. of the test enzyme in 120 minutes. Further, this effect could be reversed either by addition of $CaCl_2$ or by lowering the pH of milk containing sodium oxalate.

Effect of heating raw milk on its coagulation by the test enzyme, pepsin and rennet extract

The coagulating action of rennet is known to decrease with increase in time and temperature of heat treatment given to milk. With a view to studying the corresponding effect with the test enzyme, raw milk was heated to different temperatures by holding 100 ml. quantities in a water bath

Table 18. Effect of Addition of Sodium Oxalate to Milk on the Coagulating Activity of the Enzyme. Test enzyme: Supernatant from 18 hour litmus milk culture of C (0.4 ml/5 ml. milk)

Ml. 1 per cent sodium oxalate added/5 ml. milk*	Final milk pH	Coagulation time (min.)
None	6.63	40
0.10	6.64	58
0.15	6.65	90
0.20	6.65	120
0.20 plus 0.15 ml. of 5% lactic acid	5.70	6
0.20 plus 0.1 ml. of 1% $CaCl_2.2H_2O$	6.50	8

*milk heated to 80° C. for 15 minutes after addition of sodium oxalate

for 15 minutes after the desired temperature had been reached. The heat-treated samples were cooled by immersion in water at about 15° C. The supernatant of an 18 hour litmus milk culture of C was used as the test enzyme. Effect of addition of $CaCl_2$ to the different heat-treated samples on the enzyme activity also was studied. It will be seen from Table 19 that the test enzyme showed its maximum coagulating activity in milk heated at 70° C. Since this appeared to be a rather unusual behavior in comparison with what was

Table 19. Effect of 15-Minute Heat Treatment of Raw Milk on Its Coagulation by the Test Enzyme, Pepsin and Rennet Extract

Treatment of milk	pH of milk		Coagulation time (min.) using:					
			Test enzyme[1]		Pepsin[2]		Rennet[3]	
	No Ca	With Ca*	No Ca	With Ca*	No Ca	With Ca*	No Ca	With Ca*
Raw, unheated	6.70	6.66	53	44	10	2	14	9
Heated at 61.7° C.	6.66	6.62	51	40	10	2	14	9
Heated at 70° C.	6.65	6.63	23	20	5	1	17	9.5
Heated at 80° C.	6.63	6.60	37	24	11	2.5	45	16
Autoclaved at 14 lb.	6.52	6.50	>60	60	>60	>60	>60	>60

[1]0.4 ml. of supernatant from 18 hour litmus milk culture of C/5 ml. milk substrate

[2]0.1 ml. of 1:100 commercial pepsin preparation/5 ml. of milk substrate

[3]0.1 ml. of 1:100 rennet extract (Hansen's)/5 ml. milk substrate

*0.1 ml. of 2% $CaCl_2.2H_2O$/5 ml. milk substrate

expected on the basis of the action of rennet in milk, the coagulating activities of a commercial pepsin preparation and of rennet extract (Hansen's) were tested in a comparative way. The results of trials on milk substrate with and without added $CaCl_2$, using all three enzymes, are presented in Table 19.

The test enzyme and pepsin show their maximum coagulating power in milk heat-treated at 70° C. In case of rennet the results obtained were as would be expected; that is, a gradual increase in coagulating time with the increase in temperature of heat treatment. Autoclaved milk without added $CaCl_2$ gave no coagulation in 60 minutes with all the three enzymes tested.

Adsorption trials for separation of proteolytic from the coagulating activity

Previous studies on heat inactivation and stability of the enzyme were made in order to see if there was a possibility that such a procedure might result in the separation of the proteolytic from the coagulating activity. Failing in such an objective, some trials were made to selectively adsorb either of the fractions. Lloyd's reagent (Eli Lilly) and kaolin were the two adsorbents used at three different pH levels. The test enzyme used was the supernatant (pH 6.25) of 24 hour culture C grown on casein medium. Reaction of the test-material was adjusted to the desired pH levels

by a gradual addition of acid or alkali. For each 100 ml. of the adjusted test-material, 6.0 g. of either Lloyd's reagent or kaolin were added and mixed thoroughly. The mixture was allowed to stand for 30 minutes and centrifuged off. Both the proteolytic and the coagulating activities remained in the supernatant, although a considerable quantity of solids from the test-material was removed, particularly at pH 5.5. Results of trials made at different pH values are recorded in Table 20. When the supernatant

Table 20. Effect of Adsorbents on Enzyme Activity of Supernatant from 24 Hour Casein Medium Culture of C

Adsorbent	Adsorption carried out at:					
	pH 5.5		pH 6.5		pH 7.4	
	Coagulation**	Proteolysis*	Coagulation**	Proteolysis*	Coagulation**	Proteolysis*
Control	9	420	9	405	10	398
Lloyd's reagent (6 g./ 100 ml.)	10	390	9	375	10	368
Kaolin (6 g./ 100 ml.)	10	375	10	383	11	360

*See footnote on Table 2

**Time in minutes

obtained after this first treatment was treated a second time with the same amount of either of the two reagents, the sediment carried down both the proteolytic and coagulating activities, leaving a supernatant devoid of detectable activity of either type. The adsorption technique was not used any further for a possible separation of the two activities.

Salting out the proteolytic and coagulating activity

For convenience, the salting out procedure has been presented in Figure 5. In the case of addition of 20 g. ammonium sulphate to 80 ml. of the test-material, centrifugation was resorted to, since the precipitate formed had settled to the bottom and was too soft for scooping out as was done in the other two cases, using higher salt concentrations. Representative results of analyses of the proteolytic and the coagulating activities of the different fractions at various stages of the salting out technique followed are presented in Table 21. It will be noticed that both activities of the enzyme system were recovered by half saturation of the test material with ammonium sulphate. Recovery of the proteolytic activity on the basis of the original material, calculated on the basis of tyrosine released, was found to be 85.2 per cent. In a similar way, coagulation time in the recovered material indicated

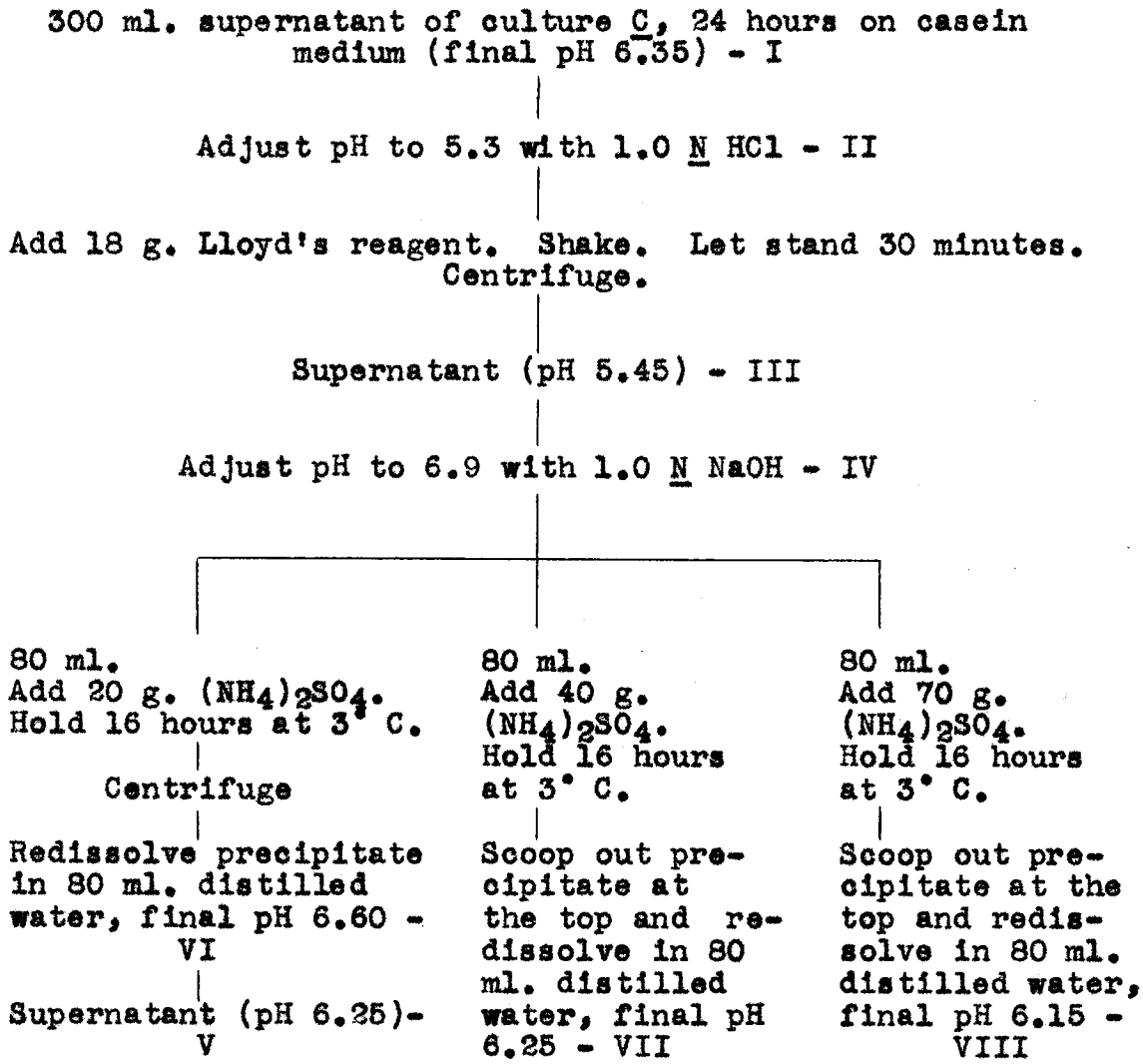

Figure 5. Salting out of the coagulating and proteolytic enzyme activities

Table 21. Coagulating and Proteolytic Enzyme Activities of Fractions Obtained by the Salting Out Procedures Outlined in Figure 5

Material tested[1]	pH	Coagulation time (min.)	Proteolysis*
I	6.35	9.5	443
II	5.30	10	420
III	5.45	9.5	435
IV	6.90	11.5	405
V	6.25	-- [2]	115
VI	6.60	16.5	278
VII	6.25	12.5	375
VIII	6.15	12.5	368

*See footnote on Table 2

[1]See Table 21

[2]This sample was not tested for coagulation since it contained a considerable amount of ammonium sulphate

a recovery of 76 per cent of the activity of the original test-material. This is in fair agreement with the values obtained for the proteolytic activity. However, the proteolytic activity in no case could be separated from the coagulating activity under the conditions of the experiments.

Study of the Endocellular Peptidases

In view of the importance of peptidases of microorganisms in the breakdown of milk proteins, their study was undertaken. The enzyme preparation used throughout was the one described in the section on Experimental Methods. The various dilutions of the "original preparation" employed will be mentioned in each case.

Influence of the pH of substrate on peptidase activity

The effect of the enzyme preparation on the buffered solutions of glycyl-L-leucine, M/30, and DL-alanylglycine, M/15, at different pH levels was studied in order to establish the point(s) or range(s) of optimum activity. In both these instances, 0.35 ml. of the "original preparation" diluted to 1.0 ml. with distilled water was added to 2.0 ml. of substrate. The enzyme-substrate mixtures were incubated in a water bath at 37.5° C. for a period of two hours. The procedure followed for estimation of the hydrolysis of the substrates has been given under Experimental Methods. Representative data for per cent hydrolysis plotted against pH of both the substrates have been given in Figure 6. In case of glycyl-L-leucine, there are two optimum pH ranges for enzyme action, one around pH 5.0 and the other in the alkaline range around pH 8.2. The activity of the enzyme

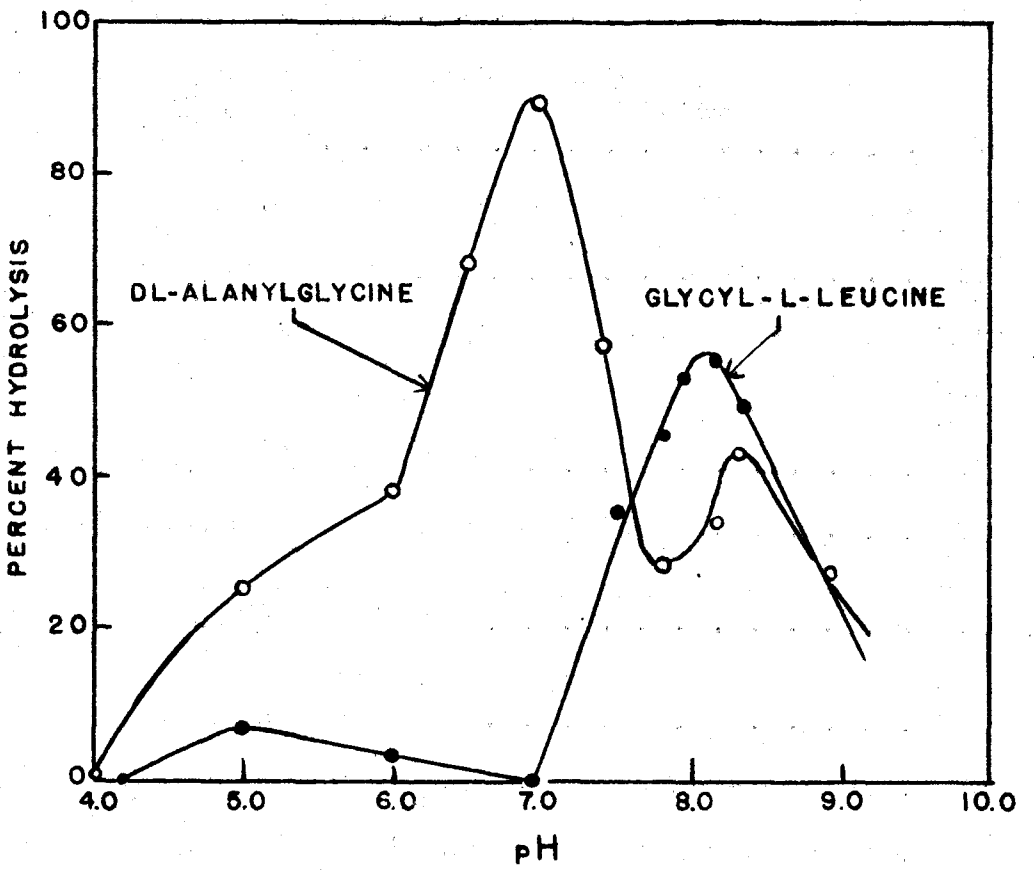

Figure 6. pH optima for enzyme preparation acting on DL-alanylglycine, M/15, and glycyl-L-leucine, M/30. (0.35 ml. "original preparation" per 2 ml. substrate incubated for 2 hours at 37.5° C., in both cases)

was considerably greater in the alkaline range. However, there was a consistent, although a small optimum at pH 5.0. No detectable activity was found either at pH 4.2 or pH 6.9. On either side of pH 8.2, there is a gradual decrease in the enzyme activity. For comparison, it might be pointed out that the enzyme activity at the optimum pH in the alkaline range is about seven times greater than the corresponding peptidase activity at the optimum pH in the acid range.

With DL-alanylglycine as a substrate, an entirely different picture is obtained. It would seem from these data that in this case also there are two distinct pH optima, one around pH 6.9 and the other in the neighborhood of pH 8.3. There also is an indication of a possible hump at pH 5.0. Between the two pH optima at 6.9 and 8.3, there seems to be a definite break around pH 7.8, although the break is not as pronounced as in the case of glycyl-L-leucine at pH 6.9. There is much greater activity of the peptidase at pH 6.9 than there is at pH 8.3.

Comparing the activity of the enzyme preparation against the two dipeptides, the results show that in the pH range of 5 to 7, there is a greater peptidase activity against DL-alanylglycine than there is for glycyl-L-leucine. It also is of interest to note that for both the substrates, pH 6.9 provides a very contrasting point, with DL-alanylglycine and glycyl-L-leucine showing maximum and minimum activities, respectively, at this point.

Effect of different cations on peptidase activity

Peptidases are known to be both activated and inhibited by several different metal cations. The effect of some of these, as well as that of cysteine hydrochloride has been studied. The compounds used for the purpose were $CoSO_4.7H_2O$, $MnSO_4$, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, $CuSO_4.5H_2O$, $FeSO_4.7H_2O$ and cysteine.HCl. Solutions of cysteine were adjusted to pH 7.0 before use. Double strength solutions of the different dilutions of the compounds and of the enzyme preparation were employed, so that on mixing the two together in equal portions, the effective concentration of both the test compound and the enzyme preparation came to the concentration desired. The combination of the test compound and the enzyme in all cases was incubated in a water bath at 37.5° C. for 45 minutes. The concentrations were so adjusted that this quantity was always 1.0 ml. After the incubation period, 2.0 ml. of the substrate were added and the peptidase activity measured by titration both at the beginning and the end of incubation period.

Glycyl-L-leucine was tested for any possible activation by seven of these compounds at pH 8.2, corresponding to its optimum pH in the alkaline range. The results (Table 22) show that Co^{++} activated in three of the higher concentrations used. Maximum activation was obtained at 10^{-3} M concentration, amounting to 85 per cent increase in activity

Table 22. Effect of Metallic Cations and Cysteine on Peptidase Hydrolysis of Glycyl-L-leucine at pH 8.2 (0.35 ml. "original preparation"/2 ml. M/30 substrate incubated 2 hr. at 37.5° C.)

Cation*	Per cent hydrolysis of one isomer using concentration of:			
	10^{-2}M	10^{-3}M	10^{-4}M	10^{-5}M
Control	54	--	--	--
$Co^{++}(SO_4)$	86	92	84	55
$Mn^{++}(SO_4)$	80	98	92	86
$Mg^{++}(SO_4)$	60	60	49	50
$Ca^{++}(Cl_2)$	28	51	51	51
$Cu^{++}(SO_4)$	15	47	55	54
$Fe^{++}(SO_4)$	10	17	58	54
Cysteine	12	36	55	54

*The salts in the effective concentration indicated were incubated with the enzyme preparation for 45 minutes at 37.5° C., prior to addition of the substrate.

over the control. In the case of Mn^{++} there was increased activity at all four concentrations tested, 10^{-3}M resulting in the maximum activity and giving an increase of 96 per cent over the control. Mg^{++} would be considered a borderline case, showing only a very slight activation in the range of 10^{-2} and 10^{-3}M. Ca^{++}, Cu^{++}, Fe^{++} and cysteine had

a very definite inhibitory action on the enzyme activity in 10^{-2}M concentration. Cu^{++}, Fe^{++} and cysteine were inhibitory in the 10^{-3}M concentration, while Ca^{++} had no noticeable effect. In the lower concentrations of 10^{-4} and 10^{-5}M none of these three ions or cysteine had any detectable effect.

Using glycyl-L-leucine as the substrate at pH 5.0, only one concentration (10^{-3}M) of Co^{++}, Mn^{++} and Mg^{++} was tried. The results are summarized in Table 23. At this pH

Table 23. Effect of Metallic Cations on Peptidase Hydrolysis of Glycyl-L-leucine at pH 5.0 (0.35 ml. "original preparation"/2 ml. M/30 substrate incubated 2 hr. at 37.5° C.)

Cation*	Per cent hydrolysis of one isomer:
Control	7
$Co^{++}(SO_4)$	49
$Mn^{++}(SO_4)$	2
$Mg^{++}(SO_4)$	8

*The salts in the effective concentration of 10^{-3}M were incubated with the enzyme preparation for 45 minutes at 37.5° C., prior to addition of the substrate

range, Co^{++} activated the enzyme quite considerably, while Mg^{++} failed to activate the enzymatic hydrolysis. Mn^{++} proved slightly inhibitory, although one would hesitate to

draw any conclusions from the datum on this point, since only one concentration of Mn^{++} was tested.

The results of similar trials, using DL-alanylglycine at pH 6.9 and pH 8.3 are presented in Tables 24 and 25, respectively. At pH 6.9, Mg^{++} increased the enzyme activity

Table 24. Effect of Metallic Cations and Cysteine on Peptidase Hydrolysis of DL-alanylglycine at pH 6.9 (0.1 ml. "original preparation"/2 ml. M/15 substrate incubated 1 hr. at 37.5° C.)

Cation*	Per cent hydrolysis of one isomer using concentration of:			
	10^{-2}M	10^{-3}M	10^{-4}M	10^{-5}M
Control	50	--	--	--
$Co^{++}(SO_4)$	48	50	50	51
$Mn^{++}(SO_4)$	50	53	51	49
$Mg^{++}(SO_4)$	75	83	68	72
$Ca^{++}(Cl_2)$	40	49	53	51
$Cu^{++}(SO_4)$	0	4	35	51
$Fe^{++}(SO_4)$	--	7	49	53
Cysteine	23	45	48	50

*The salts in the effective concentration indicated were incubated with the enzyme preparation for 45 minutes at 37.5° C., prior to addition of the substrate

in all the four concentrations employed, 10^{-3}M being the optimum concentration in this case, with an increase of 66 per cent over the control. Co^{++} and Mn^{++} failed to activate

Table 25. Effect of Metallic Cations on Peptidase Hydrolysis of DL-alanylglycine at pH 8.3 (0.1 ml. "original preparation"/2 ml. M/15 substrate incubated 1 hr. at 37.5° C.)

Cation*	Per cent hydrolysis of one isomer:
Control	40
$Co^{++}(SO_4)$	66
$Mn^{++}(SO_4)$	78
$Mg^{++}(SO_4)$	55

*The salts in the effective concentration of 10^{-3}M were incubated with the enzyme preparation for 45 minutes at 37.5° C., prior to addition of the substrate

the enzymatic hydrolysis at this pH level. These results are thus quite in contrast with the effect of these three cations on glycyl-L-leucine at pH 8.2. Ca^{++} slightly inhibited the enzyme at the 10^{-2}M level only. Similarly, cysteine had a retarding effect at 10^{-2}M concentration and a very negligible inhibition at 10^{-3}M level. Cu^{++} and Fe^{++} brought about almost complete inactivation at 10^{-2} and 10^{-3}M concentrations, with no effect at 10^{-4} and 10^{-5}M levels.

In case of DL-alanylglycine at pH 8.3, the effect of Co^{++}, Mn^{++} and Mg^{++} in the concentration of 10^{-3}M only was tested. All these three cations resulted in increased activity of the peptidase preparation. Mn^{++} showed the

highest activating effect, equivalent to 98 per cent increase over the control.

Thus using the same substrate, at two different pH levels, considerable variations in the effect of some of the cations in their action on the enzymatic activity of the peptidase preparation have been demonstrated.

Effect of proteinase on glycyl-L-leucine

An attempt was made to determine if any of the endocellular peptidases were liberated in the growth medium under normal conditions of growth and testing of the exoenzyme. Supernatant of 24 hour casein medium culture C was used as the test enzyme in this case. To 2.0 ml. of glycyl-L-leucine, M/30, at pH 8.2 and with usual buffering, were added 1.0 ml. of the test enzyme. The enzyme-substrate was incubated at 37.5° C. in a water bath for two hours. No hydrolysis of the substrate could be detected. From this it would appear that peptidases, subject of course to the limitation that only one substrate was used for testing, are not liberated in the growth medium during growth for 24 hours in a casein medium.

Effect of peptidases on casein substrate

In order to find out if the peptidase preparation had proteinase activity, 0.2 and 0.5 ml. quantitites of the

"original preparation" were added to 5.0 ml. portions of the casein substrate (1.0 per cent, pH 7.4) and incubated for three hours at 37.5° C. The usual procedure was followed for the determination of proteolysis on the casein substrate. Digestion of casein by this enzyme preparation was not obtained.

Effect of heat on peptidase activity

Specified quantities of the "original preparation" diluted to 1.0 ml. with distilled water were taken in test tubes and heated to 61.7° C. in a water bath for 30 minutes, to correspond to the normal pasteurization of milk. After this heat treatment, the tubes were plunged in cold water and transferred to a water bath at 37.5° C. To each such tube, 2.0 ml. of both of the substrates at different pH levels were added and incubated for certain period (Table 26). The results show that there was no detectable inactivation of the enzyme preparation under the conditions of the experiment.

Table 26. Effect of Heating for 30 Minutes at 61.7° C. and pH 6.60 on Peptidase Activity

Substrate	pH of substrate	Per cent hydrolysis		Remarks
		Control	Heated	
DL-alanyl-glycine, M/15	6.9	50	53	0.1 ml.* "original preparation" /2 m. substrate, 1 hr. at 37.5° C.
	8.3	40	42	
Glycyl-L-leucine, M/30	8.2	50	52	0.35 ml.* "original preparation"/2 ml. substrate, 2 hr. at 37.5° C.

*Diluted to 1.0 ml. with distilled water prior to addition of the substrate

DISCUSSION

During recent years, a great deal of progress has been made in the knowledge of the nutritional requirements of microorganisms. However, most of these studies have been directed at exploitation of the organisms as tools for the assay of amino acids, vitamins and related compounds. Some interest also has been shown in the relationship of nutrition to enzyme production.

Production of the proteolytic and coagulating enzymes by S. liquefaciens seems to be intimately tied up with growth of the organisms, as has been shown by omission of individual vitamins from the growth medium. Continued growth and production of enzymes after omission of different vitamins could be ascribed either to the ability of the organisms to synthesize these nutrients or to a possible presence of these compounds in the vitamin-test casein medium used for their growth. When the omission of a vitamin led to reduced growth of the culture, reduced enzyme production also resulted. However, the action of small amounts of vitamin B_{12} added to the vitamin-test casein medium in stimulating the production of both proteolytic and coagulating enzymes in case of culture C seems to be a deviation from the usual relationship between growth and

enzyme production. Addition of this nutrient definitely did not result in an increase in bacterial population.

That this vitamin is not an essential growth factor is borne out from the growth of this culture on a simplified amino acid medium containing no B_{12}. Further, incubation of the supernatant from a culture of C grown in vitamin-test casein medium with different amounts of B_{12} for a 30-minute period had no activating effect on the enzyme. Similarly, addition of different levels of Co^{++} to the vitamin-test casein medium used for the growth of this organism failed to duplicate the effect of B_{12}. Additions of Tween 80, sodium acetate, inositol, p-aminobenzoic acid, ascorbic acid and thiamine to the medium failed to stimulate the coagulating enzyme production either by C or by two other strains, B and E.

The adaptive character of the enzyme system has been shown by the failure of nine different strains to produce any demonstrable proteolytic or coagulating activity when grown on a simplified amino acid medium. Maximum production of both the proteolytic and coagulating enzymes occurred on vitamin-test casein medium. When grown in litmus milk, all the strains produced good amounts of the two enzyme activities. However, the enzyme activity decreased gradually with incubation periods beyond 18 hours, hardly any action being shown after 36 hours of incubation

of the cultures. This obviously is due to the instability of the two enzyme activities at low pH values, as has been found in this work. Buffering of milk was of no avail in preventing a pH drop in milk due to the growth of the organisms. However, it is conceivable that removal of fermentable sugar from milk would make it an excellent growth medium for the enzyme production in this case. In peptone broth, the tested organisms produced small amounts of these enzymes. Addition of either gelatin or casein to the broth resulted in considerable increases in both activities. Production of these enzymes in peptone broth is not surprising, since the bacterial proteinases are believed to be trypsin- or chymotrypsin-like enzymes. Chymotrypsin has been shown (Bergmann and Fruton, 1937) to hydrolyze proteins, peptones and polypeptides to the dipeptide and amino acid stage.

Increased production of the two enzyme activities due to increase in the concentration of milk solids in the growth medium possibly could be ascribed to the increase in milk proteins in the medium, in view of the similar increases in enzyme production in nutrient broth on addition of gelatin or casein. This strongly suggests the adaptive character of the two enzyme activities. Decrease in both proteolytic and coagulating enzymes as a result of addition of various types of peptones to milk for the growth of these

organisms lends additional support to the conclusion that these enzymes are adaptive in nature.

The observed fact that S. liquefaciens produces both coagulation and protein breakdown in milk commonly has been ascribed to the production of two different enzymes. However, there is no report of any work supporting such a view. Work reported in the literature has been almost entirely on the proteolytic enzymes (both exo- and endo-) produced by this organism. Some of the results obtained in this investigation are in agreement with the results of Gorini (1942), who found that maximum proteinase production in milk by Mammococcus Gorini occurred with 15-20 hours incubation. His statement that proteinase is exocellular and peptidases are endocellular also is borne out by the results obtained. According to this same worker, proteinases and polypeptidases are destroyed instantly at 100° C. In the present studies, the exocellular proteinase, as well as the coagulating activity, was destroyed at 61.7° C. in less than ten minutes. Since Gorini used gelatin as a substrate for determination of proteinase activity and employed different buffering salts, direct comparisons might be misleading, but a discrepancy in results is apparent. Gorini (1947) states that these organisms produce "common" proteinases and peptidases with optimum activity at pH 8.4 and "special" proteinases and peptidases with optimum

activity at pH 4.6. It might be pointed out that in the course of the present investigation no proteinase having its optimum at about pH 4.6 has been encountered. In case of S. liquefaciens, the optimum activity for the proteolytic enzyme activity on casein and lactalbumin lies around pH 7.4. The enzyme, however, has no such optimum for its coagulating action on milk tested within the pH range which will avoid spontaneous curdling. Within the range tested, coagulation time shortens in direct proportion to the lowering of the pH of the milk substrate. It seems reasonable to assume that the enzyme apparently would not have its optimum coagulating activity at a pH where the milk already was in a coagulated state.

Throughout the course of this investigation, irrespective of the medium used for the growth of various cultures and of the treatments given the enzyme preparation, proteolytic activity invariably has been accompanied by a proportionate coagulating activity. Similar parallelisms have been obtained in the study of heat inactivation of the two activities at different pH levels by heating to different temperatures for various lengths of time. In trials on stability of the enzyme at different pH levels at both 2-3 and 30° C., both activities responded similarly. In the same way, use of two adsorbents at different pH levels did not result in separation of the proteolytic from the coagulating activity. Negative results also were obtained

when different salt concentrations were used for salting out of the enzymes in efforts to purify and concentrate the enzymes.

Thus, if we were to apply the criteria for the unity of an enzyme as suggested by Haldane (1930), the preceding results on the effect of heat on the two activities of the enzyme, stability of the enzyme at different pH levels, adsorption and salting out techniques would seem to support the view that the proteolytic and coagulating activities of the enzyme system under investigation probably are two manifestations of the same enzyme, whose action could be likened to that of pepsin or chymotrypsin, two proteolytic enzymes with ability to coagulate milk.

Extracellular, proteolytic enzymes often have been likened to trypsin by several workers, the alkaline range of optimum activity of their enzyme preparations being the main criterion for such comparison. However, in most of these reports, many different substrates combined with diverse buffers have been employed for determination of pH optima for activity. Gelatin has been used very commonly in these studies. Since our work was carried on mainly with casein as the substrate, using phosphate as a buffer, the results of previous workers do not lend themselves to any easy comparison. However, using gelatin buffered with phosphate as the substrate, pH 7.4 was found (Wilson, 1930)

to be the optimum for activity of proteinases from B. subtilis and B. pyocyaneus (Ps. aeruginosa). No further characterization of the enzymes was made.

According to Tauber and Kleiner (1934), crude trypsin solutions clot milk only within a very limited range of their concentration, while Northrop and Kunitz (1932), using crystalline trypsin, reported that their enzyme did not clot milk. These workers also found that the optimum stability of crystalline trypsin was at pH 1.8 and that of the crude enzyme at pH 6.5. Crystalline chymotrypsin on the other hand, like trypsin, has its maximum activity against casein in the pH range 7.0 to 9.0 and has its maximum stability at pH 3.0 to 3.5. Both are reversibly or irreversibly inactivated depending on various factors. Heating at higher temperatures in acid reaction irreversibly inactivates both.

Thus, judging from the properties of these proteolytic enzymes of animal origin, it is apparent the bacterial enzymes studied in the present work or those reported by earlier workers hardly correspond to the known characteristics of either trypsin or chymotrypsin, except in a remote sense. There is a good reason to believe, on the other hand, that the bacterial proteinases, although having some broad common characteristics with the proteolytic enzymes mentioned above, could very easily form a class by themselves. There is no doubt that if due care was taken by

different workers in the field by way of using standardized techniques in their study of these enzyme systems, some very useful, comparative information could be obtained on the subject. However, such an undertaking is beyond the scope of the present work.

In view of what has been pointed out, the test enzyme of S. liquefaciens comes closest to crystalline chymotrypsin (Northrop et al., 1948). Although the enzyme tested has its optimum in alkaline reaction, it has considerable activity in the acid range. The enzyme is quite stable in the acid range when held at low temperatures. However, the action of the enzyme would be expected to be much less pronounced at low temperatures, such as those used for cheese ripening. This undoubtedly makes it a factor to be considered in the ripening process.

A rather peculiar behavior of the test enzyme and a pepsin preparation has been observed in their action on milk heated to different temperatures for 15 minutes. Milk heat-treated at 70° C. coagulated at a more rapid rate than that treated at higher or lower temperatures. This is quite contrary to the known action of rennet extract, which shows better coagulating activity on raw milk as compared to the heated milk. Two possible explanations of such a behavior seem plausible. First, heat-treatment at 70° C. for 15 minutes may bring about chemical or physical change(s)

in the milk in such a way as to make it more favorable for the action of both the test enzyme and the pepsin preparation used, while any deviation from this state brings on decreased action of the two enzymes, thus accounting for the increase in time of coagulation. Secondly, presence in milk of a factor inhibiting the milk coagulating action of the two enzymes but having no such influence on rennet is possible. This inhibiting factor would be destroyed at 70° C. for 15 minutes, thus permitting maximum action by both of these enzymes inhibited by it in raw milk. Increases in time of coagulation by either of the two enzymes, when milk is heated at higher temperatures, can be explained on the basis of decreased availability of ionized calcium in milk. It might be pointed out that addition of $CaCl_2$ to heat-treated milk resulted in considerably greater action by the test enzyme (S. liquefaciens) and pepsin, with the minimum time of coagulation obtained for milk treated at 70° C. Such a selective action of the postulated inhibitor is plausible in view of the known action of crystalline pepsin inhibitor (Northrop et al., 1948), which, while preventing both the proteolytic and coagulating activity of pepsin, has no demonstrable influence either on proteolytic activity of crystalline trypsin or milk clotting action of trypsin or commercial rennet. It would be extremely interesting to study, after isolation, the effect of such a possible inhibiting factor on the proteolytic action of the test enzyme.

Study of the endocellular peptidases of the test organism on two substrates (DL-alanylglycine and glycyl-L-leucine) suggests an extremely wide pH range of their activity. There seem to be at least two pH optima for each of the two substrates. DL-alanylglycine was hydrolyzed far more readily than glycyl-L-leucine. Zimmerman (1950) obtained similar results using the same substrates, using an endocellular preparation from S. lactis. However, this worker obtained only one pH optimum with glycyl-L-leucine and a wide pH range, strongly suggestive of two pH optima, with DL-alanylglycine as the substrate. He found no activity against either of the two substrates at pH 5.0. The results of the present investigation show inactivation of the peptidases below pH 4.2, which is in conformity with Gorini's finding that these enzymes are unstable below pH 4.5.

The results obtained for pH optima with glycyl-L-leucine are in a close proximity with the results of Gorbach (1937), who also obtained two pH optima (pH 4.8 and pH 7.0-8.4) for peptidase activity of Caseococcus Gorini, using leucylglycine as the substrate. However, he employed an incubation period of five days for determination of the enzyme activity.

Of particular interest are the results obtained for activation of the peptidase preparation with several ions. With DL-alanylglycine at pH 8.3, manganese, cobalt and

magnesium ions activated the hydrolysis of the substrate in the declining order mentioned; with the same substrate at pH 6.9, only magnesium ion could activate the enzyme, cobalt and manganese exerting no noticeable influence. Similar results were obtained by Berger *et al.* (1938b) with the peptidases of *L. mesenteroides*; they found two pH optima using these same substrates and in both cases Zn^{++} activated the enzymatic hydrolysis at the alkaline pH optimum but not in the acid pH optimum. In the present work, manganese, cobalt and magnesium activated the hydrolysis of glycyl-L-leucine at pH 8.2. At pH 5.0, cobalt was the only ion bringing about activation, magnesium ion having no effect. For both the substrates used, cysteine did not seem to have any influence.

The differences in metal activation of the enzyme preparation for the same substrate at different pH levels, seem to indicate the possibility that several enzymes, active only in specific pH ranges, may be present in the peptidase preparation used. Even with the limited number of substrates used in this study, the activity of the enzymes seems to cover a wide pH range of 4.5 to 9.0.

Use of $CaCl_2$, $Cu(SO_4)$ and $FeSO_4$ resulted in inactivation of the enzyme action when used at higher concentrations, while they had no detectable effect at lower levels. Inasmuch as these salts at higher concentrations had a

noticeable precipitation effect on the enzyme preparation during incubation for the activation trials, there is no justification for interpreting this purely chemical action as an inhibition phenomenon.

Zimmerman (1950), using a peptidase preparation from S. lactis with glycyl-L-leucine and DL-alanylglycine at pH 8.0 as the substrates, found that only Mn^{++} activated the hydrolysis of the former substrate. Other cations (Mg^{++}, Zn^{++}, Cu^{++}, and Ni^{++}) either were inhibitory or had no effect, depending on their concentration. This worker did not use Co^{++} in his studies.

It would seem that the differences in the pattern of metal activation exhibited by the organism of the present investigation and those of earlier workers, if extended to peptidases of other dairy organisms, may offer some interesting possibilities in the identification and characterization of these organisms. Only very small quantities of different metal ions are needed to activate the enzyme preparation and there seems to be quite a high level of specificity associated with the ion action.

The activity of these peptidases at low pH and their resistance to heat has a considerable potential bearing in the cheese ripening process. In conjunction with the action of proteinases, which are known to initiate the breakdown of whole proteins, these enzymes undoubtedly can be a cause

of both desirable as well as undesirable changes in milk proteins. It is, however, conceivable that the peptidase preparations can be exploited in a useful manner after further studies.

SUMMARY AND CONCLUSIONS

1. All the nine strains tested produced the greatest amounts of proteolytic and coagulating activities when grown on media containing whole protein (litmus milk, vitamin-test casein medium and casein medium). Increased concentration of milk solids resulted in increased production of both types of enzyme activity.

2. Considerable decrease in the proteolytic and coagulating activities resulted when the cultures were grown on buffered peptone broth. Addition of either gelatin or casein to this broth resulted in substantial increases in the two enzyme activities.

3. Addition of tryptone, tryptose, proteose-peptone or peptonized milk in the concentration of 1.5 per cent to litmus milk resulted in variable decreases in the production of the two activities.

4. When grown on modified Niven and Sherman's simplified amino acid medium, all of the nine strains examined failed to produce detectable amounts of proteolytic or coagulating enzyme activities, thus showing the adaptive character of the enzymes produced.

5. A drop in pH in unbuffered nutrient broth, corresponding with a similar pH drop in litmus milk resulted in

complete inactivation of the two activities in the former case.

6. Omission of individual vitamins from the vitamin-test casein medium affected both organism growth and enzyme production similarly, in no case permitting good organism growth without good enzyme production.

7. Addition of 2.0 mγ vitamin B_{12} per 5 ml. of the vitamin-test casein medium, resulted in three-fold increased in the level of proteolytic and coagulating activities by culture C. No increase in bacterial population was recorded.

8. Addition of inorganic cobalt in concentrations to substitute for the cobalt in B_{12} failed to duplicate the effect of this vitamin. Addition of vitamin B_{12} to the enzyme preparation did not increase either proteolytic or coagulating activity.

9. Vitamin B_{12} did not stimulate enzyme production by any of the eight other strains of S. liquefaciens tested.

10. Thiamine (5γ per 5 ml.) was required for the growth of strain B in the vitamin-test casein medium as well as in Niven and Sherman's amino acid medium. Culture C also required added thiamine for growth on the amino acid medium but not on the vitamin-test casein medium.

11. Addition of thiamine, Tween 80, sodium acetate, ascorbic acid, p-aminobenzoic acid and inositol to the

vitamin-test casein medium did not increase the coagulating activity by cultures C, B and E.

12. The extracellular proteolytic activity had its optimum pH at about 7.4 for digestion of both casein and lactalbumin substrates.

13. No such optimum pH for milk coagulating activity was found within the pH range attainable in milk substrate without onset of spontaneous curdling, although the coagulation time was reduced by lowering the pH.

14. The effect of heating at 50, 55 and 61.7° C. for different lengths of time was tested at various pH levels of the test enzyme. Maximum stability of both the proteolytic and coagulating activities was found to be in the pH range of 6.0 to 8.0. At 61.7° C., both the proteolytic and coagulating activities were destroyed in less than ten minutes over the entire pH range tested (5.0 to 9.0). Destruction of either of the activities always was accompanied by a corresponding inactivation of the other.

15. Both the proteolytic and coagulating enzyme activities were stable in the pH range of 5.3 to 9.0 during eight days of storage at 2.2 or 30° C.

16. Removal of ionized calcium from milk by addition of sodium oxalate resulted in complete prevention of enzyme coagulation. Addition of $CaCl_2$ or lowering of pH of milk to 5.7 restored the coagulating activity of the enzyme.

17. Peculiar susceptibility of milk heated at 70° C. for 15 minutes toward the coagulating activity of the test enzyme and to a pepsin preparation was observed. Rennet did not act in a similar way. The possibility of a chemical or a physical change in milk proteins or the presence of an enzyme-inhibiting factor in raw milk has been discussed.

18. Adsorption trials and salting out at different salt concentrations did not result in separation of the proteolytic and the coagulating activities of the test enzyme.

19. Data on heat inactivation, stability of the two enzyme activities at different pH levels and failure to separate the proteolytic enzyme from the coagulating activity by adsorption and salting out techniques all strongly suggest that the proteolytic and coagulating activities are two manifestations of a single enzyme.

20. A peptidase preparation obtained by cell grinding and autolysis was found to be active against glycyl-L-leucine and DL-alanylglycine, the latter being hydrolyzed more readily.

21. Two pH optima for peptidase activity on glycyl-L-leucine were found, one at pH 8.0 and the other at pH 5.0. Activity of the enzyme at pH 8.0 was higher compared to that at pH 5.0. The enzyme was not active at pH 4.2.

22. Two pH optima for DL-alanylglycine also were observed, one at pH 6.9 and the other around pH 8.2, with

greater activity occurring at the former pH level. There also was some indication of another optimum at pH 5.0. The substrate was not attacked at pH 4.0.

23. Hydrolysis of glycyl-L-leucine at pH 8.2 was activated by Mn^{++}, Co^{++} and Mg^{++} in the declining order stated, while hydrolysis of the same substrate at pH 5.0 was activated only by Co^{++}, with Mg^{++} and Mn^{++} exerting no influence.

24. In the case of DL-alanylglycine, Mn^{++}, Co^{++} and Mg^{++} activated the hydrolysis at pH 8.3, but at pH 6.9, Mg^{++} alone activated, with Mn^{++} and Co^{++} exerting little influence.

25. Cysteine, Fe^{++}, Cu^{++} and Ca^{++} had no activating effect for peptidases active on either of the two substrates.

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