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Proteolysis by *Lactobacillus casei*

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PROTEOLYSIS BY LACTOBACILLUS CASEI

134

by

Erling Brandsaeter

**A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY**

Major subject: Dairy Bacteriology

Approved:

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1955

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INTRODUCTION

The protein breakdown in most hard rennet cheeses is known to be brought about partly by the enzyme rennet and partly by enzymes produced by the various types of microorganisms present in the cheese, and possibly also by some milk enzymes. To what degree each of these groups of enzymes is responsible for the proteolysis which occurs in the cheese during the ripening period is not known, although much work has been done in attempting to solve this problem. The apparent lack of success probably can be ascribed in part to the fact that not enough has been known about the various factors which are involved in the process of cheese ripening. In recent years more systematic studies of the enzymes of organisms occurring in cheese have been carried out. Van der Zant and Nelson (50, 51, 52 and 53) studied the proteolytic enzymes of Streptococcus lactis. Baribo and Foster (4) compared enzyme systems obtained from various bacteria with those present in cheese, and Peterson et al. (34) tried to characterize the proteolytic enzyme systems found in cheese. A series of other workers also have contributed to the knowledge concerning this problem, but much more information is needed before all the factors involved and their relative importance in the ripening process will be known.

Lactobacillus casei is a common cheese organism found in cheddar and other hard rennet cheeses. This present work has been carried

out in order to characterize some of the proteolytic enzymes produced by L. casei, hoping that some of the information obtained may be helpful in explaining the role this organism plays in the cheese ripening process.

REVIEW OF LITERATURE

Proteolytic Activity of Lactobacilli and Their Importance
in Cheese Ripening

Many of the lactobacilli have been demonstrated to possess proteolytic enzymes. Early work by Bertrand and Weisweiler (8) showed that Lactobacillus bulgaricus in ordinary milk hydrolyzed approximately 10 per cent of the casein. Kulp and Rettger (28) reported that 2 to 6 per cent of the milk protein was broken down by Lactobacillus acidophilus and L. bulgaricus in milk incubated at 37°C. for 3 weeks. Orla-Jensen (32) concluded from his extensive studies of the lactic acid bacteria that these organisms as a rule are only poorly supplied with proteolytic enzymes, and that some strains have no effect upon either peptones or casein. On the other hand, some of these organisms, like the tetracocci and the species of S. lactis, Streptococcus cremoris, Streptococcus bovis and Streptobacterium (Lactobacillus) casei, all had proteolytic enzymes active on both peptones and casein. He also stated that the active enzymes in all these cases were endoenzymes (probably erepsin) which act only in a nearly neutral environment.

Braz and Allen (10) studied the influence of controlled pH upon protein metabolism of lactic acid bacteria in milk and found, when using mixed cultures of lactobacilli or streptococci, a marked proteolysis took place within 4 weeks. The proteolysis increased considerably when chalk was added to the milk. According to their findings, the strepto-

cocci formed mainly intermediary breakdown products of casein, such as proteoses and peptones, whereas the lactobacilli produced appreciable amounts of amino acids.

Christophersen and Thiele (12) found Lactobacillus helveticus and Lactobacillus lactis to hydrolyze casein and peptones very rapidly. Optimum hydrolysis was obtained at approximately pH 6.0, with more than 75 per cent of maximum activity remaining at pH 5.0. Leucylglycine also was hydrolyzed, the pH optimum being approximately the same as for hydrolysis of casein and peptones.

Virtanen and Lundmark (54) from their work concluded that the high numbers of Bacterium (Lactobacillus) casei ϵ in cheese one day old is strongly indicative that this organism is the most important factor in the ripening of Swiss cheese. The fact that B. casei ϵ disappeared from the cheese later on would not disprove this, since the hydrolysis of casein is a purely enzymatic reaction not depending on the presence of the living cells.

Davis et al. (15) added cultures of L. casei and Lactobacillus plantarum to the cheese starter and studied the effect upon the degree of proteolysis in cheddar cheese. They did not find any difference in the extent of protein degradation after 5 months whether the lactobacilli were added or not. After one month they found some increase in non-protein nitrogen when lactobacilli were added, but in the remaining fraction no difference was detected. Lane and Hammer (31) also studied the effect of L. casei in cheddar cheese. Among eight different strains tested, six were found to bring about more rapid and extensive decomposition

of the proteins than was the case in cheese made of pasteurized milk without any added lactobacilli. The increase in proteolysis was evident throughout the whole ripening period. On the other hand, Tittsler et al. (49) report that even though L. casei, L. plantarum, Lactobacillus brevis and Lactobacillus fermentii grew rapidly in cheddar cheese, they had little or no effect on the proteolysis or quality of the cheese. Thus, there seems to be some difference in opinion as to the role played by the lactobacilli in the cheese ripening, although the presence of lactobacilli in ripening cheese is well established, as also is indicated in works by Sherwood (38, 39 and 40) and Frazier et al. (17 and 18).

Studies with Cell-free Extracts

Proteinase activity by cell-free extracts

Tarnanen (47) was among the first ones to study the proteolytic enzyme systems of the lactobacilli by using extracts of the killed cells. He found that killed suspensions of B. casei rapidly digested casein, gelatin and peptone, the optimal conditions being at pH 6.0 and 42°C. Pretreatment of the bacterial culture with acid stronger than pH 4.5 slowly inactivated the enzymes. The enzymes were extracted from the cells with 50 per cent glycerol. The extract had the same optima of activity as the original suspension. Exposure to pH less than 3.9 or more than 9.5 rapidly inactivated the enzymes. Chloroform inactivated and phosphate inhibited the action on casein and gelatin, but not peptone. By use of adsorption on alumina, the presence of both a proteinase and a polypeptidase was shown and rough separation was obtained.

The pH optimum of the latter, which was able to split both peptones and dipeptides, occurred at approximately pH 6.8.

Baribo and Foster (4) studied the proteolytic enzymes in extracts from strains of S. lactis, L. casei and Micrococcus freudenreichii. They obtained large quantities of cells by growing them in carrot-liver extract broth for 7 to 9 days at 30°C. The cells were collected by centrifugation and washed repeatedly with distilled water. Enzyme extract was obtained by mixing cell paste with glass glow beads and shaking it in a carbon dioxide atmosphere at low temperature. After shaking, 2 ml. of distilled water per gram of cells were added, the mixture placed in a refrigerator for 24 hours and then centrifuged to remove the cell debris. The obtained extracts were tested on sodium caseinate substrate and compared with enzyme extracts obtained from cheese. The cheese extract gave two temperature optima, one at 45°C. and one between 11 and 18°C. The extract from L. casei also showed two temperature optima, the lower one corresponding fairly well with the lower optimum of the cheese enzyme. The pH optimum for proteinase activity by cell-free extract from L. casei tested at 30°C. occurred at pH 6.0 to 6.5, whereas at 11°C. the optimum was close to pH 7.0. The latter corresponds fairly well with the pH optimum of the cheese enzyme at 11°C. Several reducing agents activated the proteinase from L. casei. Metal ions generally were inhibitory, except that calcium showed some activation.

Amundstad (1) studied the proteolytic activities of two strains of L. casei and found their pH optimum for hydrolysis of a 3 per cent

peptone solution to occur between pH 6.0 and 6.6. Tests on casein as a substrate showed that the rate of increase in soluble nitrogen was highest at pH 6.0, whereas the rate of increase in amino nitrogen was highest at pH 6.5. The results furthermore showed that the proteolysis took place over a wide pH range. At pH 5.0 the rate of increase in soluble nitrogen was still 50 per cent of the maximum rate, and at pH 7.2, 75 per cent of maximum rate. For increase in amino nitrogen the corresponding values were 40 and 64, respectively, indicating that the breakdown into the lower components occurred at a somewhat narrower pH range.

Bacterial peptidases

An extensive survey of bacterial peptidases was made by Berger, Johnson and Peterson (6). This survey includes a table listing bacterial peptidases reported in the literature. The table includes the pH optima for the peptidase activity in the cases where they were determined. According to this information, most of the bacterial peptidases have pH optima in the range from pH 7.0 to 8.5. There are a few reports showing bacterial peptidases which have a more acidic pH optimum. Gorbach and Ulm (20) and Gorbach (19) found peptidases active against leucylglycine which had an optimum at pH 4.8. More recently Van der Zant and Nelson (52), in working with S. lactis, found the pH range for maximum peptidase activity on a series of peptides to occur between pH 7.0 and 9.0. Zimmerman (57) found peptidases from S. lactis to have two pH optima for hydrolysis of alanylglycine; one at approximately pH 6.0 and the other at pH 8.0. Dudani (16) obtained similar results

for peptidases from enterococci when tested on alanylglycine as a substrate, whereas the hydrolysis of glycylleucine was found to be maximum at pH 5.0 and 8.0. In some work reported by Berger et al. (6), Lactobacillus pentosus was found to produce acidopeptidases hydrolyzing leucylglycylglycine at a maximum rate at pH 5.5, and Popionibacterium pentosaceum produced a peptidase with maximum activity on alanylglycine occurring at pH 5.5 to 6.0.

Metal activation of peptidases has been reported by several workers. Berger et al. (6), using four different organisms, found leucylpeptidase-like enzymes whose hydrolysis of leucylglycylglycine was activated by manganese ions in a concentration of 0.003 M. The same workers (5) found that Leuconostoc mesenteroides produced peptidases whose action on leucylglycine, alanylglycine, alanylglycylglycine and diglycylglycine was activated by zinc ions at pH 8.0, whereas the hydrolysis of glycylglycine and leucylglycylglycine was inhibited under the same conditions. At pH 5.8 the hydrolysis of all these peptides was inhibited by zinc ions.

Dudani (16) found that the hydrolysis of glycylleucine at pH 8.2 by peptidases from Streptococcus liquefaciens was activated by cobalt, manganese and magnesium ions. Calcium, copper and ferrous ions were inhibitory when present in a concentration of 0.01 M. At pH 5.0 cobalt strongly activated the hydrolysis of glycylleucine. The hydrolysis of alanylglycine at pH 6.9 was activated by magnesium and inhibited by copper and ferrous ions; cobalt showed no effect. At pH 8.3, cobalt,

manganese and magnesium ions activated the hydrolysis of alanylglycine. Zimmerman (57) reported that the hydrolysis of glycylleucine by peptidase from S. lactis was activated by manganese at pH 8.0. The hydrolysis of alanylglycine at pH 8.0 was inhibited by manganese. Copper, zinc, magnesium and nickel ions were in both cases somewhat inhibitory. Van der Zant and Nelson (52), working with cell-free extracts of S. lactis, also found manganese to activate the hydrolysis of glycylleucine, whereas magnesium and cobalt activated the hydrolysis of alanylglycine at pH 8.0. Gorini and Fromageot (21), in studying the peptidases of Micrococcus lysodeikticus, found that fractional precipitation with acetone separated the endopeptidases into inactive protein fractions which were reactivated by manganese ions.

Schormüller et al. (36) studied the peptidases from extracts of sour milk cheese. They found a dipeptidase with maximum activity at pH 7.8, and an aminopeptidase and a carboxypeptidase with maximum activities at pH 7.0 to 7.2. Studies on metal activation revealed that manganese activated the hydrolysis of leucylglycine, leucylglycylglycine and benzoylglycylglycine. Cobalt strongly activated the hydrolysis of leucylglycine and leucylglycylglycine, but inhibited the hydrolysis of benzoylglycylglycine. Magnesium inhibited the hydrolysis of leucylglycine, but otherwise had little effect on the hydrolysis of the other substrates.

Non-bacterial peptidases

Metal ion activation of peptidases from sources other than bacteria

also has been reported. Smith and Spackman (44) found manganese or magnesium ions to be essential for the activity of a leucine aminopeptidase obtained from swine kidney. Smith (41) studied the glycylglycine dipeptidase from skeletal muscle and human uterus and found it to be an extremely specific enzyme which was strongly activated by cobalt and somewhat less activated by manganese ions at pH 8.0. He suggested that the specific coordination compound formed between cobalt ions and glycylglycine may be specific to the enzyme. In a theoretical study of the enzymic hydrolysis of glycylglycine in the presence of cobalt, Goudot (22) postulated that a covalent complex is formed between glycylglycine and cobalt; the reaction occurred between the complex cobalt-enzyme and three molecules of glycylglycine. The energy of activation was calculated to be between 12 and 14 Kcal. per mol. The role of metallic ions in the enzymatic hydrolysis of peptides is discussed by Smith (43) in an extensive review covering the general properties and specificities of peptidases.

Isolation and Purification of Enzymes

There is a great volume of literature published in the field of enzyme isolation and purification. To give a review of all the work done in this field will not be attempted here; only a few articles of special interest in connection with the present study will be included. Among recent reviews of general interest on this topic may be mentioned one by Schwimmer and Pardee (37) on the principles and procedures in the isolation of enzymes, and one by Zittle (58) on adsorption studies of

enzymes and other proteins.

With respect to proteolytic enzymes of microbial origin, Tarnanen (47) used an alumina gel to obtain a rough separation of enzymes in a cell-free extract of L. casei containing a proteinase and a polypeptidase. Van der Zant and Nelson (52), in attempting to isolate a peptidase from a cell-free extract of S. lactis, applied fractional precipitation with ammonium sulfate or ethanol and subsequent adsorption with kaolin or alumina C γ . They succeeded in obtaining a fraction which hydrolyzed alanyl-glycine, but had no effect on casein or glycyllucine. A somewhat similar procedure was used by Korkeas et al. (27) in isolating a "malic" enzyme, but the latter workers introduced another step during the course of the purification in that they removed nucleic acids and inactive protein by precipitation with protamine at pH 5.5. They also used tricalcium phosphate as the adsorbent.

Wetter (56) studied the proteolytic enzyme systems of Mortierella renispora. By moving boundary electrophoresis they found a protease concentrate obtained from the culture medium of M. renispora to be made up of a number of proteins. Filter paper electrophoresis demonstrated that two of the proteins were able to hydrolyze denatured hemoglobin. One active component had a negative and the other a positive mobility in phosphate buffer at pH 6.8, ionic strength 0.1. Because of the difference in electrophoretic properties, the two components could be separated by zone electrophoresis. Though yields were low when filter paper was used, the use of potato starch as the supporting medium resulted in excellent recoveries.

Zone electrophoresis has recently been used much in separating enzymes and other proteins. Kunkel and Slater (29) point out that the complete separation of components, rather than the partial separation occurring in the unpacked channel of the Tiselius cell, makes this method useful for preparative purposes. The use of filter paper as the supporting medium has its limitations in that it has low capacity, it may adsorb some components and cause trailing, and furthermore, elution of the material from filter paper may be difficult and give low recoveries. Kunkel and Slater point out the advantages of using starch as the supporting medium. It is favorable because of the homogenous packing achievable, the lack of adsorption, good recoveries and relative low electrosmosis. Tiselius (48) also mentions other supporting media which can be used, such as powdered paper, glass beads and gel. He concludes that under suitable conditions, i. e. minimizing of adsorption phenomena and electrosmosis, mobilities and isoelectric pH-values can be determined by this method. The theories on paper electrophoresis, which basically are the same as for zone electrophoresis on other supporting media, have recently been published in a book by Block et al. (9).

MATERIALS AND METHODS

Designations and Sources of the Cultures and Their Propagation

L. casei strains 7, 25, 28 and 142 used in this study were obtained from stock cultures carried by this laboratory, and they were not specially identified in connection with this work. Three of these cultures had been obtained originally from the University of Wisconsin.

The cultures were grown in glucose-yeast extract milk (13), incubated at 37° C. and transferred daily for 3 days prior to each trial. Stock cultures initially were kept in glucose-yeast extract milk containing 10 per cent powdered chalk. In this medium the cultures could be kept viable for at least 3 months when stored at 2° C. During the later part of this work, actively growing culture was inoculated into a series of tubes with glucose-yeast extract milk and immediately frozen. A 10 per cent inoculum was used. Prior to each trial, the frozen culture was incubated at 36° C. for 24 hours, and two additional transfers were carried out before the culture was used. It was believed that this would give less possibility of variations in the characteristics of the cultures.

Preparation of Media

During the course of this work the following media were used.

Sterile skim milk

Fresh skim milk from the College Creamery was sterilized at 120°C. for 13 minutes.

Plate count agar

For the enumeration of the lactobacilli the plate count agar described by Briggs (11) was used. This medium had the following composition:

V-8 juice	200 ml.
Neopeptone	15 g.
Dextrose	20 g.
NaCl	5 g.
Tween 80	1 g.
Yeast extract	6 g.
Soluble starch	0.5 g.
Bacto agar	15 g.
Distilled water to	1000 ml.

The medium was adjusted to pH 6.8 and autoclaved at 15 lb./sq. in. for 20 minutes.

Modified Briggs medium

A modification of Briggs medium (11) for growth of lactobacilli was used to obtain large amounts of cells for use in enzyme studies. The composition of this medium was as follows:

V-8 juice	200 ml.
Bacto peptone	5 g.
Washed casein	15 g.
Tween 80	1 g.
Peptonized milk	5 g.
Yeast extract	5 g.
Glucose	2 g.
Soluble starch	0.5 g.
NaCl	2 g.
$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (0.1 M)	13.8 g.
Distilled water to	1000 ml.

In the preparation of this medium batches of six liters were usually made up. Ninety g. of washed casein (Fischer Scientific Company) were suspended in 3 l. of distilled water, the reaction adjusted to approximately pH 10.5 with 5 N sodium hydroxide, and then steamed for 15 minutes. The other ingredients were dissolved in the rest of the water, mixed with the casein suspension and the reaction adjusted to pH 6.85. The medium was distributed in 1.5 l. portions into 2 l. erlenmeyer flasks and sterilized at 120°C. for 13 minutes.

Preparation of Cell-free Extracts of L. casei

Growing and harvesting cells

Large amounts of cells for preparation of cell-free extracts were obtained by growing the organisms in four 1.5 l. batches of the modified

Briggs medium. The inoculated medium was incubated at 35°C. for a period of time sufficient to decrease the pH of the medium from pH 6.85 to pH 5.8, usually 22 to 24 hours.

The cells were harvested by centrifugation in a Sharpless Super centrifuge operated at 30,000 r.p.m. The cells were washed twice in cold 0.03 M phosphate buffer at pH 7.0, recovered by centrifugation in a refrigerated centrifuge and stored at 0°C.

Preparation of cell-free extract by sonic vibration

The harvested cells were dispersed in 0.03 M phosphate buffer at pH 7.0. The ratio of wet cells to buffer was approximately 1:2. The cells were disrupted by sonic vibration in the chilled cup of a Raytheon 9 kilocycle magnetostriction oscillator. Disintegration was carried out on 20 ml. samples and for a time of 30 minutes. The cell debris was centrifuged out and washed once with buffer solution. Approximately 3 ml. of enzyme extract was obtained per gram of wet cells. The cells obtained from growth in 6 l. of the medium yielded 120 to 140 ml. of cell-free extract.

Preparation of Substrates for Testing Proteolytic Activities by Cell-free Extract

Substrate for proteinase activity

Two per cent suspension of casein was used for testing the proteinase activity by cell-free extracts. The substrate was prepared by suspending 4 g. of casein (Fisher Scientific Company) in approximately

100 ml. distilled water, adjusting to pH 10.0 with sodium hydroxide and heating in a steamer for 10 to 15 minutes. After cooling, 10 ml. of a 0.5 M composite buffer (acetate, phosphate and borate) was added. The substrate was adjusted to desired pH and made up to 200 ml. with distilled water. Merthiolate was added as preservative at a rate of 1 mg. to 40 ml. of substrate. The substrate was stored at 2°C.

Substrate for peptidase activity

The peptidase activity was tested on the following substrates:

M/30 glycylglycine, diglycylglycine, glycyl-L-leucine, glycyl-L-tyrosine, glycyl-L-tryptophane and carbobenzoxyglycyl-L-phenylalanine, M/15 glycyl-DL-alanine, DL-leucylglycine, DL-alanylglycine, DL-alanylglycylglycine, DL-leucylglycylglycine and on M/7.5 DL-alanyl-DL-alanine.

The substrates were usually made up in 100 ml. quantities by dissolving the required amount of peptide in approximately 75 ml. distilled water and 10 ml. of 0.5 M composite buffer. The substrates were adjusted to desired pH with 1 N sodium hydroxide or 1 N hydrochloric acid and made up to 100 ml. with distilled water. Merthiolate was added as a preservative at the rate of 2.5 mg. per 100 ml. The substrates were stored at 2°C.

Methods for Determination of Proteolytic Activities

Determination of soluble nitrogen in skim milk

Nitrogen soluble in 2 per cent trichloroacetic acid was determined by a method developed for examination of cheese serum by Lane and Hammer (30), with the exception that a 5 g. sample was used instead of the 1 ml. sample of cheese serum used by Lane and Hammer. To a 5 g. sample were added 40 ml. of distilled water and 5 ml. of a 20 per cent solution of trichloroacetic acid. The mixture was stirred vigorously and kept overnight in the refrigerator, filtered through paper and the precipitate washed with 2 per cent solution of trichloroacetic acid. The amount of nitrogen in the filtrate was determined by the Kjeldahl-Gunning-Arnold (3) method. Digestion was carried out by using 10 g. of sodium sulfate, 2 g. of copper sulfate and 20 ml. of concentrated sulfuric acid. After digestion was completed and the solution cooled, 200 ml. of distilled water, 100 ml. of sodium hydroxide solution (480 g. of 76 per cent sodium hydroxide per liter) and a few zinc pellets were added to each flask. Distillate was caught in 50 ml. 0.05 N hydrochloric acid containing 8 drops of methyl red-methylene blue indicator (26). Back titrations were carried out using 0.05 N sodium hydroxide. For total nitrogen, 5 g. samples were pipetted directly into 500 ml. Kjeldahl flasks.

Colorimetric method for determination of proteolysis in milk

Proteolysis also was determined by a method worked out by Anson (2) and modified by Hull (25) for the purpose of measuring the hydrolysis of milk proteins. The method is based on the increase in a trichloroacetic acid filtrate of tyrosine and tryptophane available to react with the Folin-Ciocalteu reagent to form a blue color.

For determination of proteolysis by growing cells in milk, 1 ml. of active culture was inoculated into 100 ml. of sterile skim milk in screw-cap bottles and mixed thoroughly; 5 ml. samples were transferred into sterile screw-cap tubes. The tubes then were incubated at the desired temperature for the desired length of time. At the end of the incubation period, 2 ml. of distilled water and 10 ml. of a 0.72 N trichloroacetic acid solution were added while agitating the test tube to mix the milk and acid. The tube was allowed to stand for 10 minutes before filtering through paper. One ml. of the filtrate was added to a 50 ml. erlenmeyer flask, followed by 4 ml. of distilled water and 10 ml. of sodium carbonate-quadrates reagent (75 g. of anhydrous sodium carbonate and 10 g. of sodium tetraphosphate dissolved in distilled water and made up to 500 ml.). The contents of the flasks were mixed thoroughly before 3 ml. of the Folin-Ciocalteu reagent was added under continuous shaking. Five minutes were allowed for the blue color to reach a maximum before reading was performed in a Klett-Summerson photoelectric colorimeter, using a 645 m μ wavelength filter. Controls containing 1 ml. of distilled

water instead of the trichloroacetic acid filtrate were run at zero time and the readings of the controls were subtracted from the sample readings. A standard tyrosine curve, showing the colorimetric readings for various tyrosine concentrations was used to convert the sample reading into its tyrosine equivalent (Figure 1), and the results are given as γ tyrosine per ml. of substrate. The procedure used for color development of the standard solution was the same as that employed for the milk samples. The tyrosine used in the standard solution was L-tyrosine (Merck), recrystallized and dried.

Colorimetric method for determination of proteolysis in casein suspension

Proteinase activity by cell-free extracts was determined on 2 per cent casein suspension by the colorimetric method described above, except that 5 ml. of casein suspension, 1 ml. of enzyme extract and 1 ml. of distilled water made up the digestion mixture, and 0.6 N trichloroacetic acid was used for precipitation.

Determination of non-casein nitrogen

Non-casein nitrogen in the 2 per cent casein substrate was determined according to Rowland's method (35) for the test on milk. The method was modified for the present purpose and carried out as follows: Ten ml. of enzyme-substrate mixture was pipetted into a 150 ml. erlenmeyer flask. Thirty ml. of water at 45°C. and 1 ml. of 10 per cent acetic acid were added, mixed and kept at 45°C. for 10 minutes before 1 ml. of 1 N sodium acetate was added. The

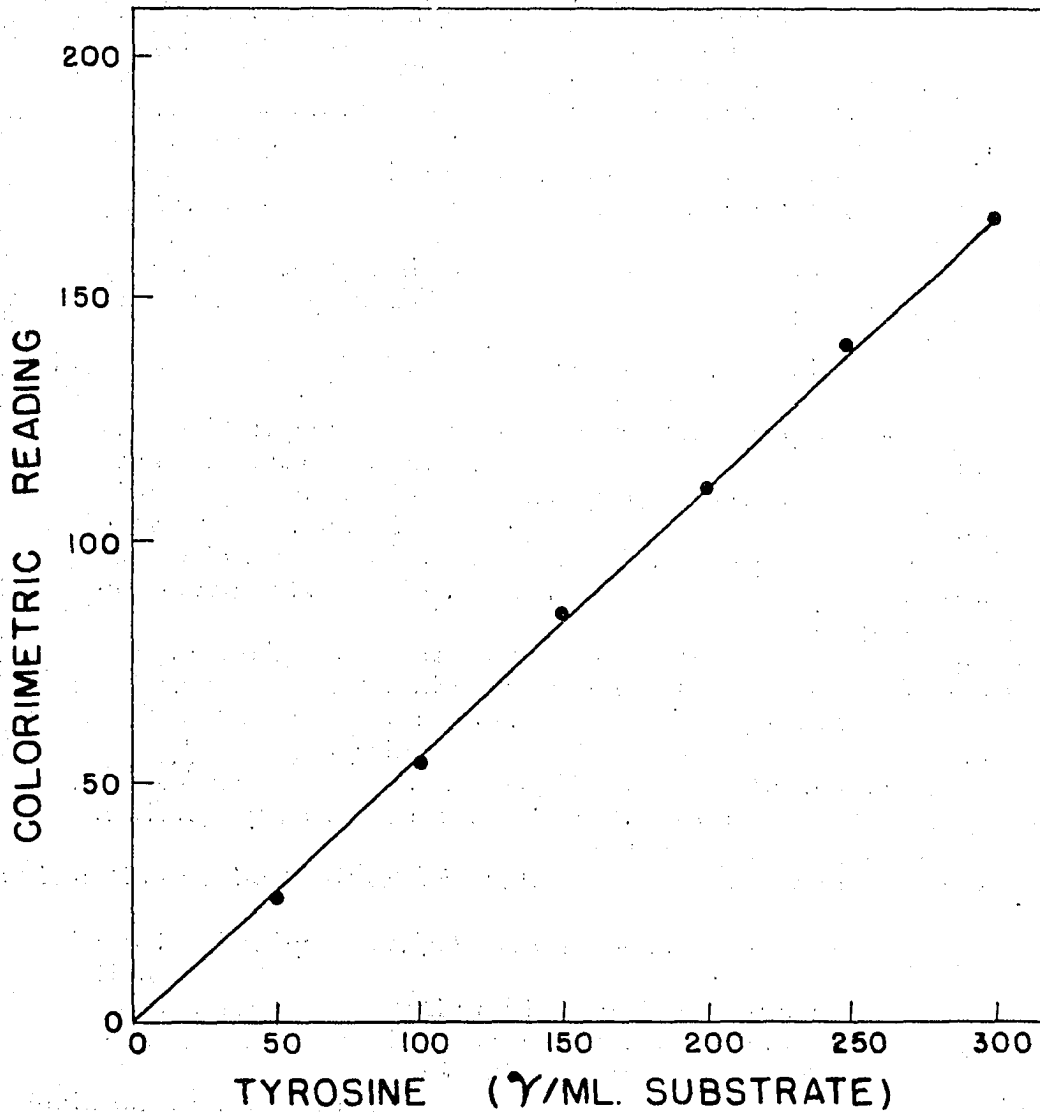


Figure 1. Standard curve relating micrograms of tyrosine to color produced by Folin-Giocalteau reagent

mixture was allowed to cool and then was filtered through No. 40, 9 cm. Whatman paper. The precipitate was washed with water containing 1 ml. of 10 per cent acetic acid and 1 ml. of 1 N sodium acetate per 40 ml. Nitrogen in the total amount of filtrate was determined according to Kjeldahl's method.

Determination of non-protein nitrogen

Determination of non-protein nitrogen in the 2 per cent casein substrate was carried out by using a modification of Rowland's method (35) for determination of non-protein nitrogen in milk. To 10 ml. of enzyme-substrate mixture in a 150 ml. erlenmeyer flask were added 35 ml. of distilled water and 5 ml. of 20 per cent trichloroacetic acid. The mixture was thoroughly shaken, and after 10 minutes filtered through No. 40, 9 cm. Whatman paper. The precipitate was washed with 2 per cent trichloroacetic acid. Nitrogen in the whole filtrate was determined by Kjeldahl's method.

Determination of amino nitrogen

Determination of amino nitrogen was carried out on the filtrate after precipitation with phosphotungstic acid according to a method described by Orla-Jensen and Plattner (33). The method was modified for the present purpose. Ten ml. of enzyme-substrate mixture was pipetted into a 150 ml. erlenmeyer flask, followed by 20 ml. of distilled water. Five ml. of 25 per cent sulfuric acid was added and followed by 4 ml. of 10 per cent phosphotungstic acid. The mixture

was thoroughly shaken, and after 10 minutes filtered through No. 40, 9 cm. Whatman paper. Nitrogen in the whole filtrate was determined by the Kjeldahl's method.

Determination of peptidase activity by cell-free extract

The reaction mixture, in screw-cap test tubes, consisted of 2.5 ml. of substrate plus cell-free extract (usually 0.2 ml.). The cell-free extract was added when the substrate reached the temperature of incubation. The reaction mixture was incubated for the desired length of time (usually 1 hour) in a water bath at the desired temperature. Hydrolysis of the peptides was determined by titration of the carboxyl groups as described by Grassmann and Heyde (23). At the beginning and at the end of the incubation period, 1 ml. of the reaction mixture was titrated with 0.05 N ethanolic potassium hydroxide, using a 0.1 per cent ethanolic solution of thymolphthalein as indicator. The titrations were carried out in 20x175 mm. test tubes, and the endpoint was determined by the color in the mixture of 1 ml. of 0.1 M composite buffer at pH 9.8, six drops of indicator and 10 ml. absolute ethanol. The titration was carried out as follows: One ml. of reaction mixture was pipetted into a test tube followed by six drops of the indicator solution. A solution of 0.05 N ethanolic potassium hydroxide then was run in from a micro burette until the contents turned blue. At this stage, absolute ethanol, nine times the volume to be titrated, was added, which was accompanied by

disappearance of the blue color. More ethanolic potassium hydroxide was added until the endpoint was reached.

Per cent hydrolysis was calculated on the basis of the difference in titer at the beginning and the end of the incubation period. The results are expressed as per cent hydrolysis of one optical isomer. In case of substrate of the DL-form, which was made up to M/15, it was assumed that only the L-form was attacked by the enzyme, and the calculation was therefore based on a substrate concentration of M/30.

Other Determinations

Determination of titratable acidity

Titratable acidity was determined on 9 ml. samples, using 5 drops of 2 per cent phenolphthalein as indicator and titrating with 0.1 N sodium hydroxide.

Determination of pH

A Beckman Model G glass electrode pH meter was used for all pH determinations.

Determination of bacterial count

Bacterial counts were carried out on special agar as described earlier in this section. Plates were incubated at 35°C. for 48 hours.

Purification and Separation of Proteolytic Enzymes

Protein nitrogen determination by the biuret test

In the course of purification of the cell-free extracts, protein determinations were carried out, using the biuret test as proposed by Weichselbaum (55). A standard curve was constructed (Figure 2) using the protein of a cell-free extract of L. casei. The amount of protein nitrogen was determined by Kjeldahl's method. In carrying out the test, from 0.1 to 0.5 ml. of cell-free extract, depending on the concentration, was pipetted into a test tube and made up to 5 ml. with a saline solution followed by 5 ml. of Biuret reagent. After mixing, the tube was incubated at 32°C. for 30 minutes before the developed color was measured in a Klett-Summerson photoelectric colorimeter, using a filter of 555 m μ wavelength. The amount of protein nitrogen was read from the standard curve after a blank determination had been subtracted.

Preparation of adsorbents

Tricalcium phosphate gel was prepared according to the directions given by Sumner and O'Kane (46). Alumina C γ , prepared according to the directions given in Bertho-Grassmann's Biochemisches Praktikum (7), also was used. The latter adsorbent was available at this laboratory and was not made up in connection with this work.

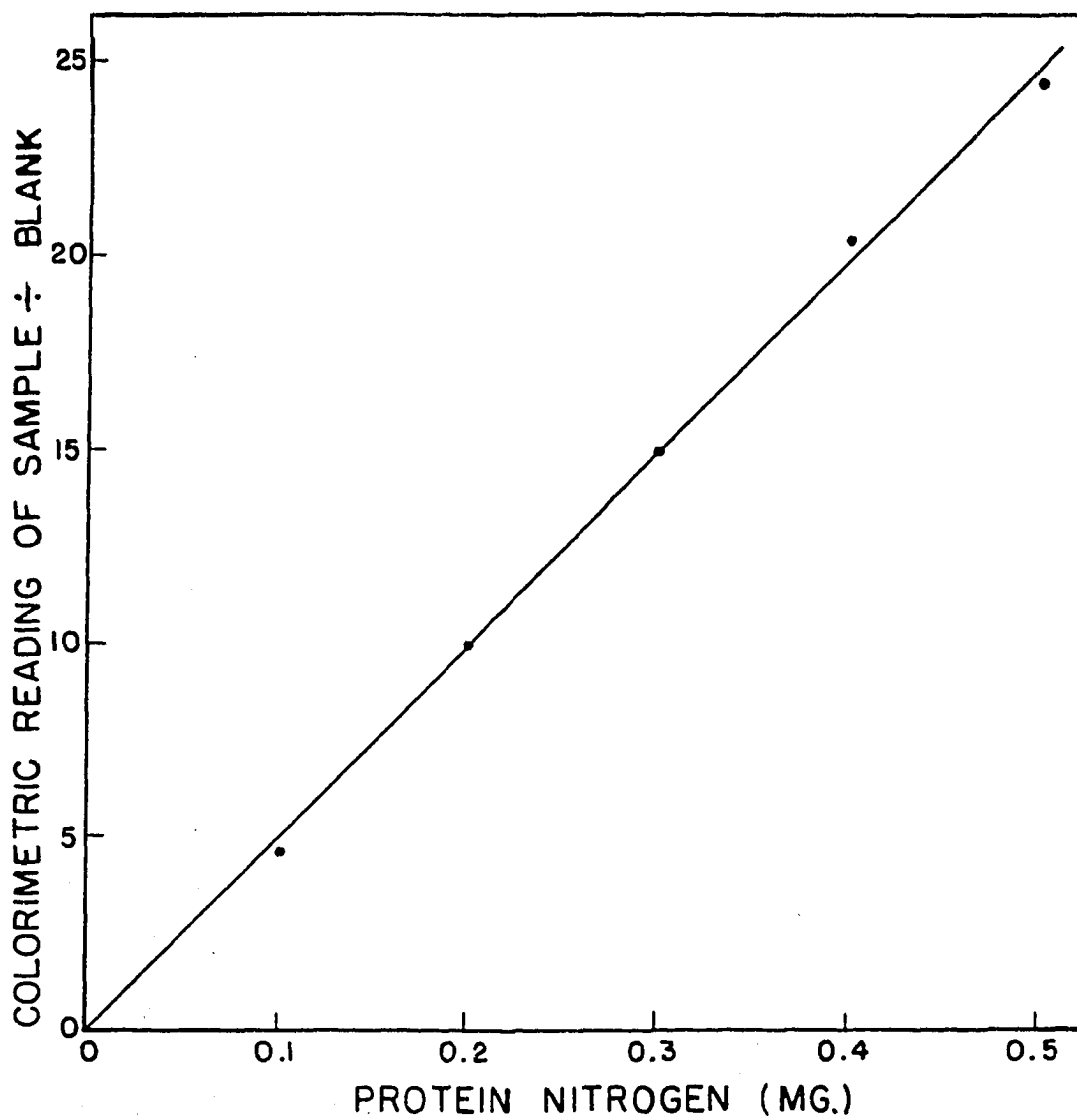


Figure 2. Standard curve relating milligrams of protein nitrogen to color produced by biuret reagent

Preparation of protamine solution

Protamine solution for precipitation of nucleic acids was made up of protamine sulfate (Nutritional Biochemicals Company). The protamine sulfate was dissolved in distilled water at the rate of 20 mg./ml., and adjusted to pH 5.5 with sulfuric acid. In order to completely dissolve the protamine, the solution was heated in a steamer for 10 minutes.

Spectrophotometric examinations

All spectrophotometric examinations were carried out with a Beckman Model DU spectrophotometer.

Zone electrophoresis of cell-free extract

Separation of proteolytic enzyme activities from the cell-free extracts was attempted by zone electrophoresis, using potato starch as the supporting medium. The experiments were carried out with an E-C 401 electrophoresis apparatus and using 0.1 M phosphate buffer. The relative mobilities of the various components in the cell-free extract were determined by experimental runs on paper strips at different pH levels. Based on the results obtained, it was decided to make runs on a starch block at pH 6.65, although some runs also were carried out at other pH levels. The starch block was made up by mixing potato starch (J. T. Baker Chemical Company) with buffer at an approximate ratio of 1:1 to give a viscous starch suspension. The suspension then was poured into the rectangular migration chamber of the electrophoresis apparatus which

was covered at the bottom and along the sides with non-wettable paper and at the ends with filter paper. Excess moisture was dried out with heavy filter paper. The enzyme was added by cutting out a rectangular segment approximately 7 mm. wide across the middle of the block, and replacing it with a barely moist mixture of cell-free extract and starch. Approximately 18 ml. of cell-free extract could be applied this way. The block then was covered by the non-wettable paper, and on top of that a glass plate. Contact of starch block with the buffer solution was obtained with cellulose sponges. Ice water was circulated through the glass plates on both sides of the starch block in order to minimize heat inactivation of the enzymes. Movements of the proteinaceous material were followed by pressing a filter paper strip against the top of the starch block at certain time intervals. The filter paper was then dried and sprayed with a ninhydrin solution (0.2 per cent ninhydrin in butanol saturated with water and containing 3 per cent phenol) and color developed over a hot plate. The experiments were usually run at 200 volts and long enough for the fastest moving proteinaceous material to travel to the ends of the starch block, usually from 20 to 30 hours. The block was then cut into segments according to the zones of proteinaceous materials showing on the filter paper strips. Each segment was mixed with a minimum amount of ice-cold distilled water and filtered through paper in a Buchner funnel under suction. Good recoveries could be obtained by this method. The recovered material was in some cases concentrated by keeping it in dialysis bags in front of a fan. This was

usually carried out in a cold room, although some runs were made at room temperature (approximately 25°C.).

Determination of Protein Degradation in Cheese

Determination of total, soluble and formal nitrogen in cheese was carried out according to the method described by Sode-Mogensen (45).

Total nitrogen

Twelve and one half g. of finely ground cheese were weighed into 150 ml. glass beakers. The cheese was dissolved in 50 ml. of 0.5 M sodium citrate solution, alkaline enough to give red color with phenolphthalein. The temperature was kept at 40 to 45°C. and 60 ml. of distilled water of the same temperature was added. This solution was kept in a water bath at 40°C. for 30 minutes, and stirred in order to get any remaining cheese particles into solution. The contents of the beaker then was transferred into a 250 ml. volumetric flask, cooled to 20°C. and made up to the mark with distilled water. Total nitrogen was determined by Kjeldahl's method, using 10 ml. of the cheese suspension, which then represented 0.5 g. of cheese.

Soluble nitrogen

Two hundred ml. of the cheese-citrate suspension in a 250 ml. volumetric flask was cooled to approximately 15°C. in running tap water. Non-soluble nitrogen was then precipitated by adding 20 ml. of 1.41 N

hydrochloric acid under vigorous stirring. This brings the pH of the solution down to $\text{pH } 4.40 \pm 0.05$. The flask then was filled up to the mark with distilled water and the contents filtered through paper. The first filtrate coming through was refiltered in order to obtain as clear a filtrate as possible. Soluble nitrogen was determined by the Kjeldahl's method on 25 ml. of the filtrate which represented the soluble nitrogen in 1 g. of cheese.

Formol nitrogen

To 25 ml. of the hydrochloric acid filtrate was added five drops of 2 per cent phenolphthalein and 2.6 ml. of 1 N sodium hydroxide. Final titration was carried out with 0.1 N sodium hydroxide. The endpoint was determined by comparison with the color of a solution consisting of 25 ml. water and three drops of a 0.01 per cent aqueous fuchsin solution. After the endpoint was reached, 10 ml. of a 30 to 40 per cent formaldehyde solution and two drops of the phenolphthalein solution were added, and the solution was again titrated with 0.1 M sodium hydroxide to the same endpoint. The amount of 0.1 M sodium hydroxide used in the second titration minus the amount used for neutralizing 10 ml. of the formaldehyde solution was the formol titer per 1 g. of cheese. Formol nitrogen as per cent of total nitrogen then could be computed on the basis of the formol titer and the hydrochloric acid consumption from the determination of total nitrogen, keeping in mind that the values obtained for total nitrogen represented 0.5 g. of cheese whereas the formol titer represented 1 g. of cheese. The value

obtained is, according to Sode-Mogensen (45), called reduced formol nitrogen as per cent of total nitrogen ($\% N_{fr}$), and was found to correspond closely to the values obtained for amino nitrogen by the phosphotungstic acid method.

EXPERIMENTAL RESULTS

Proteolysis by L. casei Grown in MilkTest for activity of four strains of L. casei

One ml. quantities of actively growing cultures of L. casei strains 7, 25, 28 and 142 were inoculated separately into screw-cap bottles containing 100 ml. of sterile skim milk. After thorough mixing, 5 ml. samples were transferred with sterile pipettes into each of ten tubes. For each of the cultures, one bottle and three tubes were incubated at temperatures of 32, 37 and 45°C.

The samples were tested after 0, 24, 48 and 192 hours. Bacterial counts were obtained on Briggs (11) plate count agar for lactobacilli. Plates were incubated at 35°C. for 48 hours. Nitrogen soluble in 2 per cent trichloroacetic acid was determined by Kjeldahl's method on 5 g. samples taken from the screw-cap bottles after thorough mixing. The test was run as described under Materials and Methods. Proteolysis, as measured by the test for tyrosine plus tryptophane, was determined on the samples in the screw-cap tubes, according to the method described earlier. Titratable acidity was run on 9 ml. samples taken from the screw-cap bottles. The results are given in Table 1. According to these results, L. casei 25 is definitely the most proteolytic culture.

Table 1. Growth rate and biochemical activity of four strains of L. casei grown in skim milk

Incub. temp. °C.	Strain no.	Bacterial count ^a after an incubation time (hr.) of:		Soluble N ^b as per cent of total N after an incubation time (hr.) of:			Tyrosine in γ/ml. ^c after an incubation time (hr.) of:			Per cent acidity ^d after an incubation time (hr.) of:		
		24	48	24	48	192	24	48	192	24	48	192
32	7	90x10 ⁷	25x10 ⁸	11.3	11.1	14.1	77	100	133	0.24	0.38	1.47
	25	10x10 ⁷	75x10 ⁸	13.6	14.5	17.7	95	172	220	0.76	1.20	1.65
	28	80x10 ⁷	12x10 ⁸	10.1	10.6	11.1	70	70	93	0.39	0.60	0.79
	142	60x10 ⁷	29x10 ⁸	10.8	10.8	14.3	70	76	185	0.27	0.61	1.54
37	7	60x10 ⁷	28x10 ⁸	10.8	11.3	14.8	77	82	153	0.25	0.47	1.38
	25	25x10 ⁷	77x10 ⁸	14.3	14.3	14.8	138	139	213	1.14	1.56	1.78
	28	70x10 ⁸	32x10 ⁸	11.6	11.3	11.3	75	80	95	0.43	0.61	0.78
	142	12x10 ⁸	20x10 ⁸	11.3	11.6	15.5	75	90	198	0.36	0.83	1.44
45	7	20x10 ⁵	33x10 ⁶	11.7	11.1	13.9	75	70	78	0.27	0.18	1.55
	25	50x10 ⁵	14x10 ⁵	12.9	12.7	14.8	143	125	180	1.32	1.60	1.60
	28	10x10 ⁷	34x10 ⁷	11.3	10.4	14.6	70	69	77	0.18	0.18	1.51
	142	18x10 ⁷	46x10 ⁷	-	10.1	14.6	88	80	162	0.31	0.72	1.18

^aBacterial count at zero time: 10x10⁵.

^bSoluble nitrogen as per cent of total nitrogen at zero time: 10.0.

^cTyrosine γ/ml. at zero time: 70.

^dPer cent acidity at zero time: 0.17.

It grew rapidly at all three temperatures and possessed higher proteolytic activity and produced more acid than any of the other strains. Strain 28 was the least active. The difference in activity between strain 25 and the other three strains was particularly noticeable in the early stages of the incubation period. Strains 7 and 28 definitely showed poor growth at 45°C. during the first 48 hours, although by 192 hours acid production and soluble nitrogen values were high, but tyrosine values remained low. There was not much difference in proteolytic activity by strains 25 and 142 at the three temperatures, strain 25 showing maximum proteolytic activity at 32°C., whereas strain 142 caused the highest degree of proteolysis at 37°C. As a result of these tests, strains 25 and 142 were selected for further studies.

Effect of chalk on proteolysis by *L. casei* grown in skim milk

Active cultures of *L. casei* strains 25 and 142 were each inoculated into two screw-cap bottles containing 159 ml. of sterile skim milk. Ten per cent powdered chalk was added to one bottle but not to the other. A 1 per cent inoculum was used. The cultures were incubated at 35°C. for a period of 228 hours. The samples were shaken (120 shakes per minute) throughout the incubation period by an automatic shaking device in order to keep the chalk in suspension. Samples were taken at intervals for determination of pH and for total, soluble and insoluble nitrogen. Nitrogen was determined on 5 g. samples as described under Materials and Methods, and the results are shown in Table 2. In comparing the activity of the two strains when no chalk was added, it is

Table 2. Influence of chalk on proteolysis by L. casei grown in skim milk at 35°C.

Strain no.	Time	pH	Nitrogen in 5 g. of milk (expressed as ml. 0.05 N HCl)			Sol. N as per cent of total N
			Sol. N	Insol. N	Total N	
25	0	6.50	4.1	40.7	44.6	9.2
	12	5.60	4.3	39.8	44.5	9.7
	36	5.15	5.8	37.7	43.8	13.2
	60	4.95	6.7	36.2	43.3	15.4
	84	4.90	7.0	35.3	42.2	16.5
	228	4.40	8.5	31.0	39.8	21.3
25 plus chalk	0	6.60	4.0	39.3	43.6	9.2
	12	5.75	4.4	38.8	43.5	10.1
	36	5.65	5.5	37.7	42.8	12.8
	60	5.50	6.2	34.8	40.9	15.2
	84	5.35	7.1	32.7	39.9	17.2
	228	5.05	10.1	25.8	36.1	28.0
142	0	6.50	4.0	40.7	44.5	9.0
	12	6.25	4.1	40.3	44.0	9.3
	36	5.10	4.3	40.0	44.0	9.8
	60	4.30	5.0	39.0	43.4	11.5
	84	4.15	5.6	38.3	43.5	12.9
	228	3.25	6.2	38.6	44.2	14.0
142 plus chalk	0	6.60	3.9	39.0	43.2	9.2
	12	6.40	4.0	38.8	43.0	9.3
	36	5.60	-	-	-	-
	60	5.25	4.8	36.2	40.9	11.7
	84	5.25	-	-	-	-
	228	5.00	9.7	28.6	38.6	25.1

seen that strain 142 brought about the greatest drop in pH whereas strain 25 showed the highest proteolytic activity. The slower drop in pH by strain 25 might be caused by the higher degree of proteolysis by this strain, rather than a slower acid production. The addition of chalk to the cultures did not have any effect on the degree of proteolysis during the first 60 hours. A considerably higher degree of proteolysis was observed at 228 hours in the cultures which contained the added chalk. In the cultures where chalk was added the pH did not drop below 5.0. According to the results obtained here, the rate of proteolysis apparently was not greatly reduced before pH dropped down to 4.5 or below. Because of its high proteolytic activity, strain 25 has been used throughout the remainder of this work.

Effect of pH on proteolysis by *L. casei* grown in skim milk

Five tenths of a ml. of actively growing cultures of *L. casei* 25 was inoculated into each of four screw-cap bottles containing 350 ml. of sterilized skim milk. Prior to the inoculation, the milk in the four bottles was adjusted to pH's 4.5, 5.5, 6.5 and 7.5, respectively. The inoculated samples, together with one uninoculated control, were incubated at 36°C. Electrometric titrations were carried out on 10 ml. samples every four to five hours, using 0.1 N sodium hydroxide. The pH was maintained by additions of 1 N sodium hydroxide, the amounts being computed on the basis of the electrometric titrations. The pH of the samples was then checked and a final adjustment was carried out if necessary. The volumes of the samples were carefully kept track of.

The difference in the amounts of sodium hydroxide added was corrected for by adding sterile distilled water. The amount of neutralizer and water in the milk after 96 hours was approximately one fourth of the remaining volume. The samples were incubated for a period of 96 hours, and the pH usually dropped from 0.3 to 0.6 of a unit between each adjustment, depending somewhat on the pH to which they were adjusted. Total and soluble nitrogen were determined in 5 g. samples by the Kjeldahl method after 0, 24, 48 and 96 hours for the inoculated samples. The control was tested after 0 and 96 hours. Soluble nitrogen as per cent of total nitrogen was calculated on the basis of duplicate Kjeldahl tests and the results are shown in Figure 3.

The rate of proteolysis was highest at pH 6.5, but the proteolytic activity of the culture growing at pH 5.5 was still high. The difference in rate of proteolysis at pH 6.5 and 5.5 was most pronounced during the first 48 hours of the incubation period. From then on little difference was observed between these two samples. The rates of proteolysis at pH 4.5 and 7.5 were nearly the same and much slower than at pH 6.5 and 5.5.

Effect of temperature on proteolysis by *L. casei* grown in skim milk

Five tenths of a ml. of actively growing culture of *L. casei* was inoculated into each of four screw-cap bottles containing 400 ml. of sterilized skim milk. The pH was adjusted to 6.5 and the four bottles were incubated at 32, 36, 40 and 44°C., respectively. The pH of the

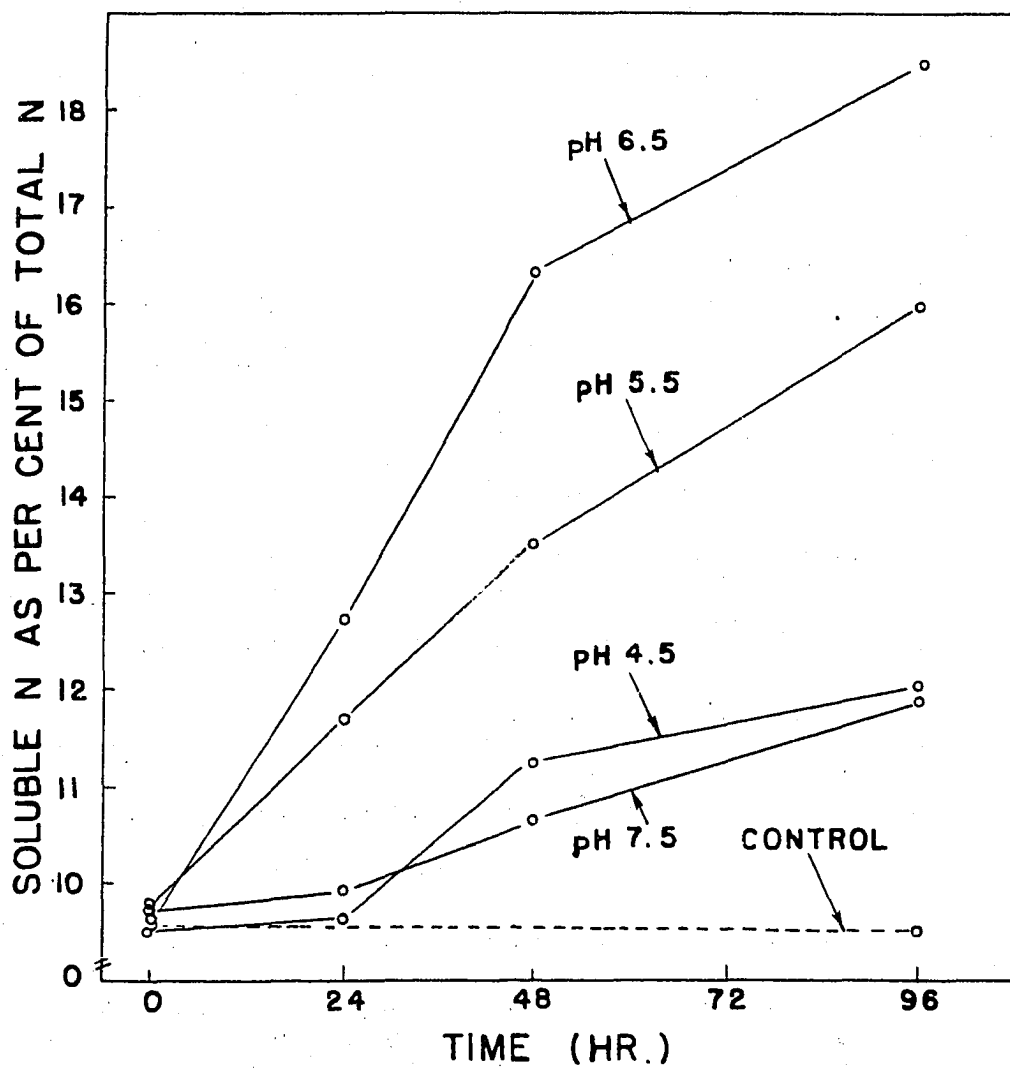


Figure 3. Effect of pH on the proteolysis by *L. casei* grown in milk at 36°C. (based upon per cent of nitrogen soluble in 2 per cent trichloroacetic acid)

samples was maintained as described under the previous experiment. Total and soluble nitrogen were determined on 5 g. samples by duplicate Kjeldahl tests. Tests were run after 0, 24, 48 and 96 hours, and the results shown in Figure 4 are given as soluble nitrogen in per cent of total nitrogen.

The rate of proteolysis at 36, 40 and 44° C. was nearly the same during the first 24 hours of the incubation period. From then on the rate increased with decreasing temperature. The proteolytic activity at 32° C. was slower during the early stages of the incubation period, but at this temperature the rate of proteolysis was almost constant throughout the test period, resulting in the highest degree of proteolysis after 96 hours. The degree of proteolysis after 96 hours decreased with increasing temperature.

Proteinase Activity by Cell-free Extract of L. casei 25

Effect of time of sonic vibration on the activity of cell-free extract of L. casei

Cell-free extracts were prepared as described under Materials and Methods, with the exception that disintegration was carried out on 20 ml. samples of the cell suspension for 20, 30 and 40 minutes. The proteolytic activities of the cell-free extracts then were tested according to the tyrosine-tryptophane method as described earlier, except that 0.1 and 0.5 ml. of the cell-free extracts were used in 5 ml. of 2 per cent casein suspension at pH 6.5. The samples were incubated at 35° C. for 24 hours. The results given as increase in tyrosine are shown in Table 3.

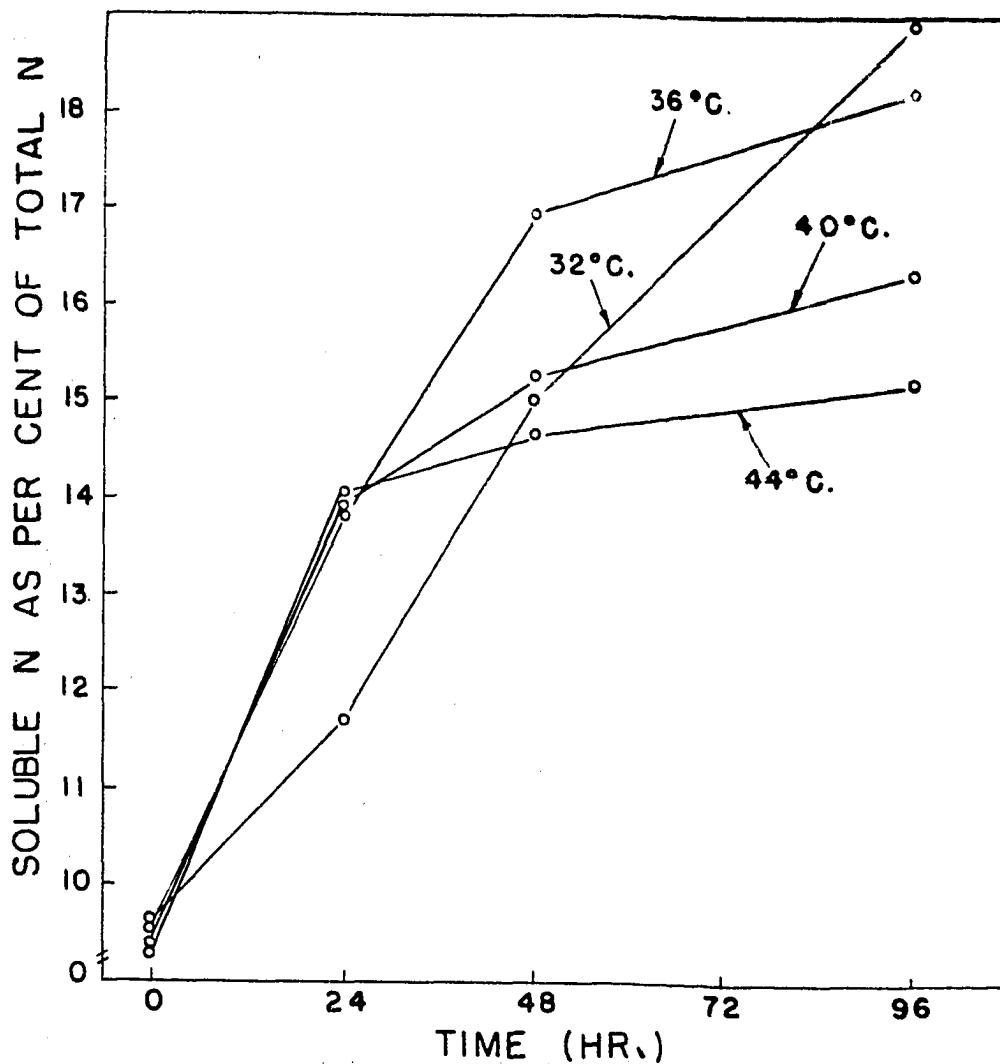


Figure 4. Effect of temperature on the proteolysis by *L. casei* grown in milk at pH 6.5 (based upon per cent of nitrogen soluble in 2 per cent trichloroacetic acid)

Table 3. Effect of time of sonic vibration on the activity of cell-free extract of L. casei

Time of vibration (min.)	Increase in tyrosine (γ /ml. substrate) ^a	
	0.1 ml. extract	0.5 ml. extract
20	10	52
30	16	52
40	16	56

^aCell-free extracts in 5 ml. of 2 per cent casein suspension at pH 6.5, incubated at 35°C. for 24 hours.

According to the obtained results there was little difference in the activity of the cell-free extracts with the variations in time used in disintegrating the cells. Throughout the remainder of this work disintegration was carried out for 30 minutes.

Effect of storage on the activity of frozen cell-free extract with and without added cysteine

Cell-free extract at pH 7.0 was distributed in 3 ml. portions in ten 10x75 mm. tubes. One drop of 2 M cysteine hydrochloride (pH 7.0) was added to five of the tubes. The tubes then were stoppered and stored in a freezer chest at -16°C. Activity tests of the cell-free extracts were run after various lengths of time by testing the increase in tyrosine and tryptophane produced by 0.5 ml. of cell-free extract as described under Materials and Methods. The results obtained are shown in Table 4.

Table 4. Effect of storage at -16°C . on the activity of cell-free extract with and without added cysteine

Time of storage (days)	Increase in tyrosine (γ /ml. substrate) ^a	
	No cysteine added	Cysteine added
0	53	53
1	52	53
3	44	-
7	30	32
14	26	30

^aFive tenths of a ml. of cell-free extract in 5 ml. of a 2 per cent casein suspension at pH 6.5; incubated at 35°C . for 24 hours.

The proteolytic activity of the frozen cell-free extract decreased rather rapidly during the first week of storage, whereas during the second week less change in activity was observed. Cysteine, in the concentration used here, had little effect on the proteolytic activity and the keeping time of the cell-free extract.

Effect of pH at different temperatures on proteinase activity by cell-free extract of *L. casei*

Two per cent casein suspension in batches of 100 ml. was adjusted to desired pH with 5 N sodium hydroxide or 5 N hydrochloric acid, and distributed in 5 ml. portions into screw-cap tubes. One ml. of cell-free extract and 1 ml. of sterilized distilled water were added, and the tubes incubated at desired temperatures. Proteinase activity was tested for after 12 and 24 hours by the colorimetric method for tyrosine and

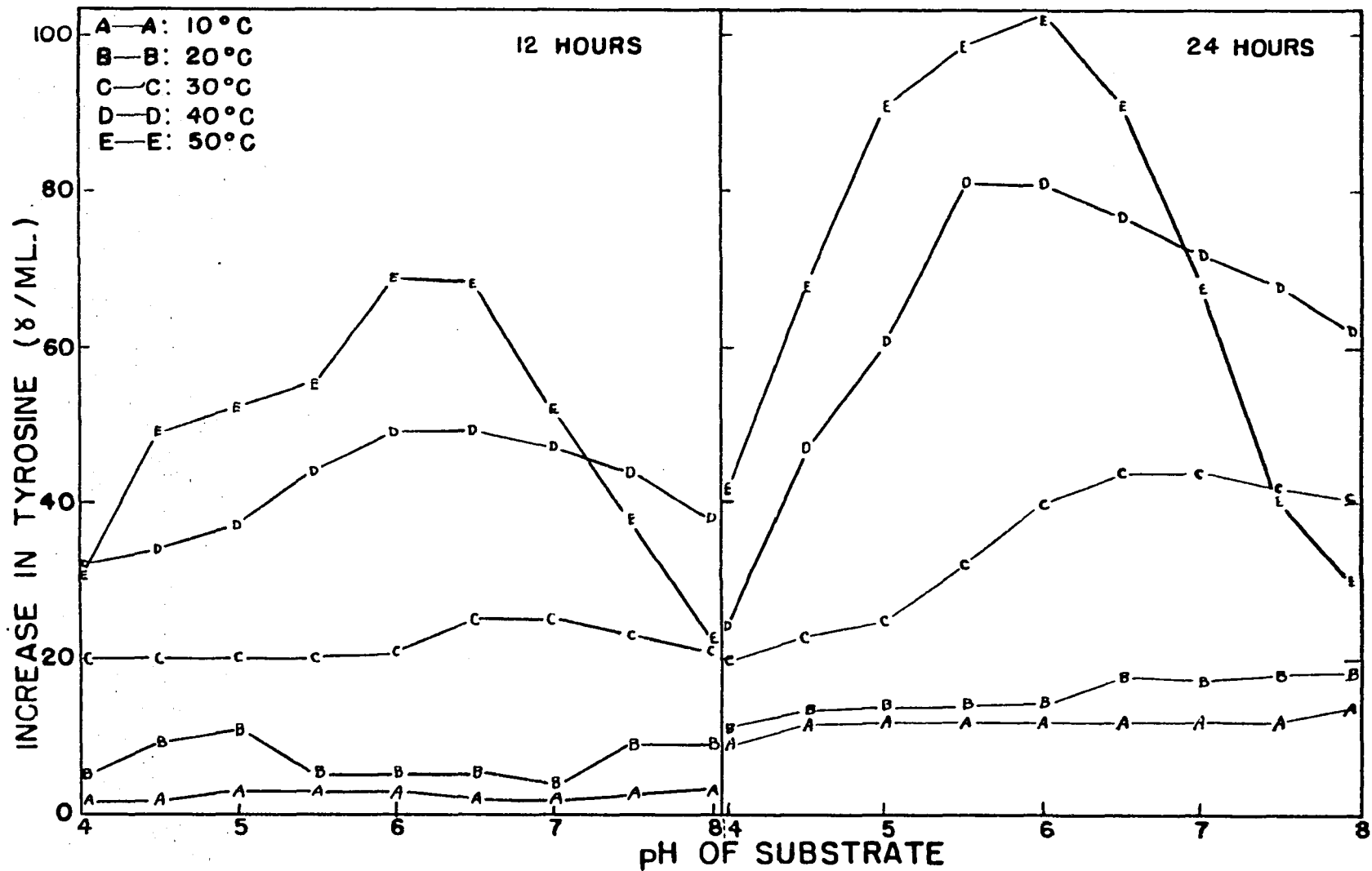
tryptophane, as described under Materials and Methods. The results obtained are shown in Figure 5. These results were reproduced fairly closely in a second run.

The optimum pH for proteinase activity varied with varying temperatures. At 50°C. the optimum occurred at near pH 6.0, but tended to move towards a neutral pH as the temperature was lowered. The slight shift in pH optimum towards a more acid pH with increased time of holding, and the rapid decrease in proteinase activity at 50°C. as the reaction was raised above pH 6.5, may indicate that the enzyme was less stable at the higher pH levels. The highest level of activity was obtained at 50°C., which was the highest temperature used in this experiment. Little difference in the proteolytic activity was observed as the temperature was raised from 10 to 20°C.

Effect of temperature on proteinase activity by cell-free extract of *L. casei*

Tests were run on 5 ml. of 2 per cent casein suspension at pH 6.0. One ml. of cell-free extract and 1 ml. of sterile distilled water were added, and the screw-cap tubes containing the digestion mixture were incubated at the desired temperatures. Proteinase activity was determined after 12 and 24 hours by the colorimetric method for tyrosine and tryptophane. The results are shown in Figure 6.

The optimum temperature for proteinase activity at pH 6.0 was found to occur at approximately 50°C. The activity decreased rapidly as the temperature was raised to 55°C., this being more pronounced after 24 than after 12 hours of incubation. The rate of hydrolysis was almost doubled for each 10°C. raise in temperature up to 45°C. Between 45



44

Figure 5. Effect of pH at different temperatures on proteinase activity by cell-free extract of *L. casei* tested on 2 per cent casein suspension

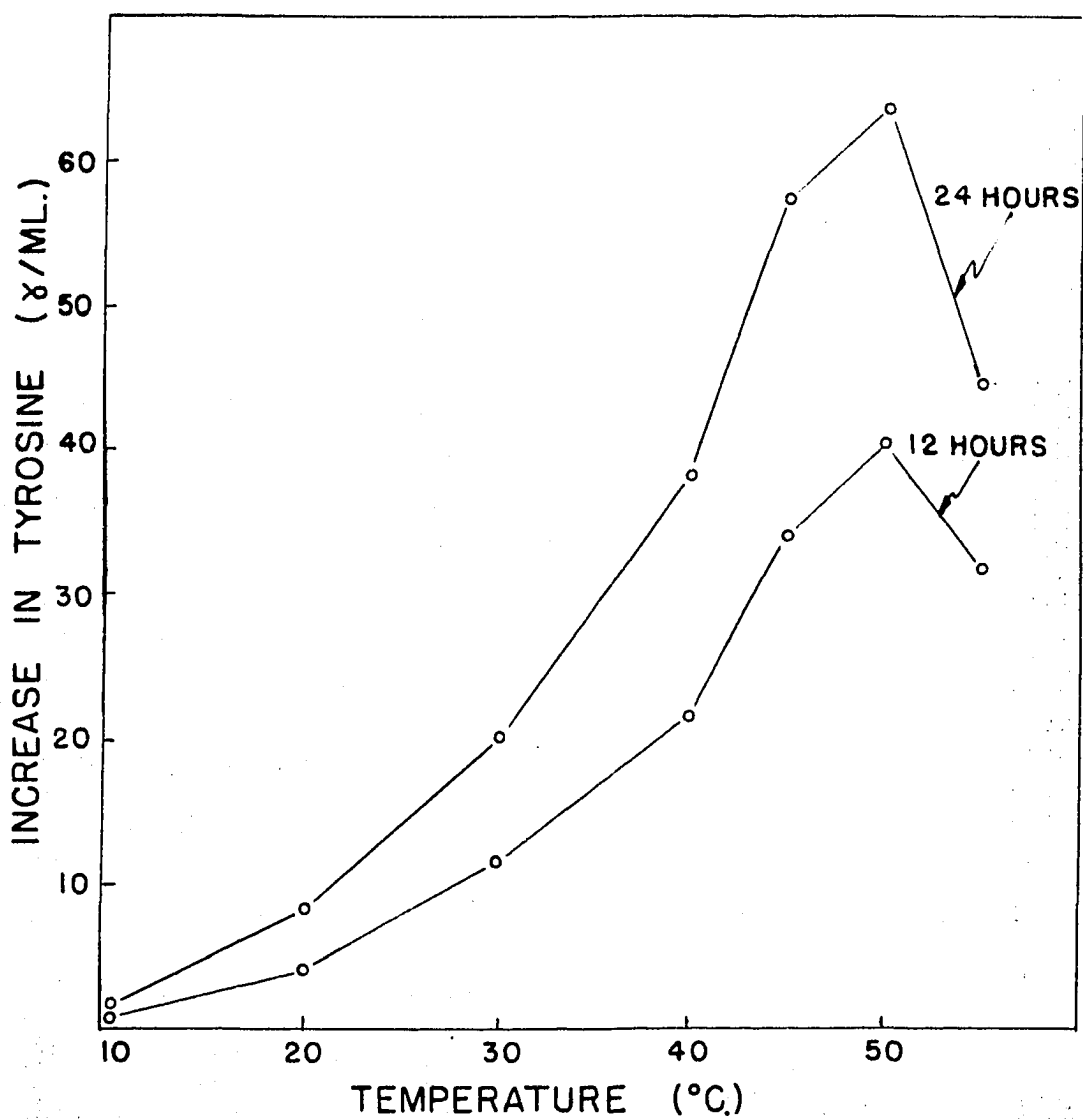


Figure 6. Effect of temperature on proteinase activity by cell-free extract of *L. casei* tested on 2 per cent casein suspension at pH 6.0

and 50°C. the increase in rate of hydrolysis was less, probably due to the fact that some of the enzyme was being denatured at this temperature.

Effect of time and temperature on proteinase activity by cell-free extract of *L. casei*

The digestion mixture and the pH of the digestion mixture in this experiment were the same as in the previous one. Proteinase activity was tested for by the colorimetric method for tyrosine and tryptophane, and the results are shown in Figure 7. The rate of hydrolysis was almost constant throughout the incubation period for temperatures up to 40°C. Above this temperature a decrease in the rate of proteolysis was observed as the time was prolonged. This decrease probably was due to partial heat inactivation of the enzyme, rather than to changes in the substrate concentration. The initial rate of hydrolysis at 55°C. was much higher than at 40°C., but the degree of hydrolysis at these two temperatures was approximately the same after a period of 30 hours.

Effect of time on the increase in non-casein, non-protein and amino nitrogen by cell-free extract of *L. casei*

The digestion mixture consisted of 180 ml. of 2 per cent casein suspension and 20 ml. of cell-free extract; the reaction was adjusted to pH 6.0. The mixture was incubated in a water bath at 45°C. Total nitrogen, non-casein nitrogen, non-protein nitrogen and amino nitrogen were determined on 10 ml. samples after 0, 12 and 24 hours, according to the methods given in the section on Materials and Methods. The results shown in Figure 8A are the average of duplicate runs which paralleled

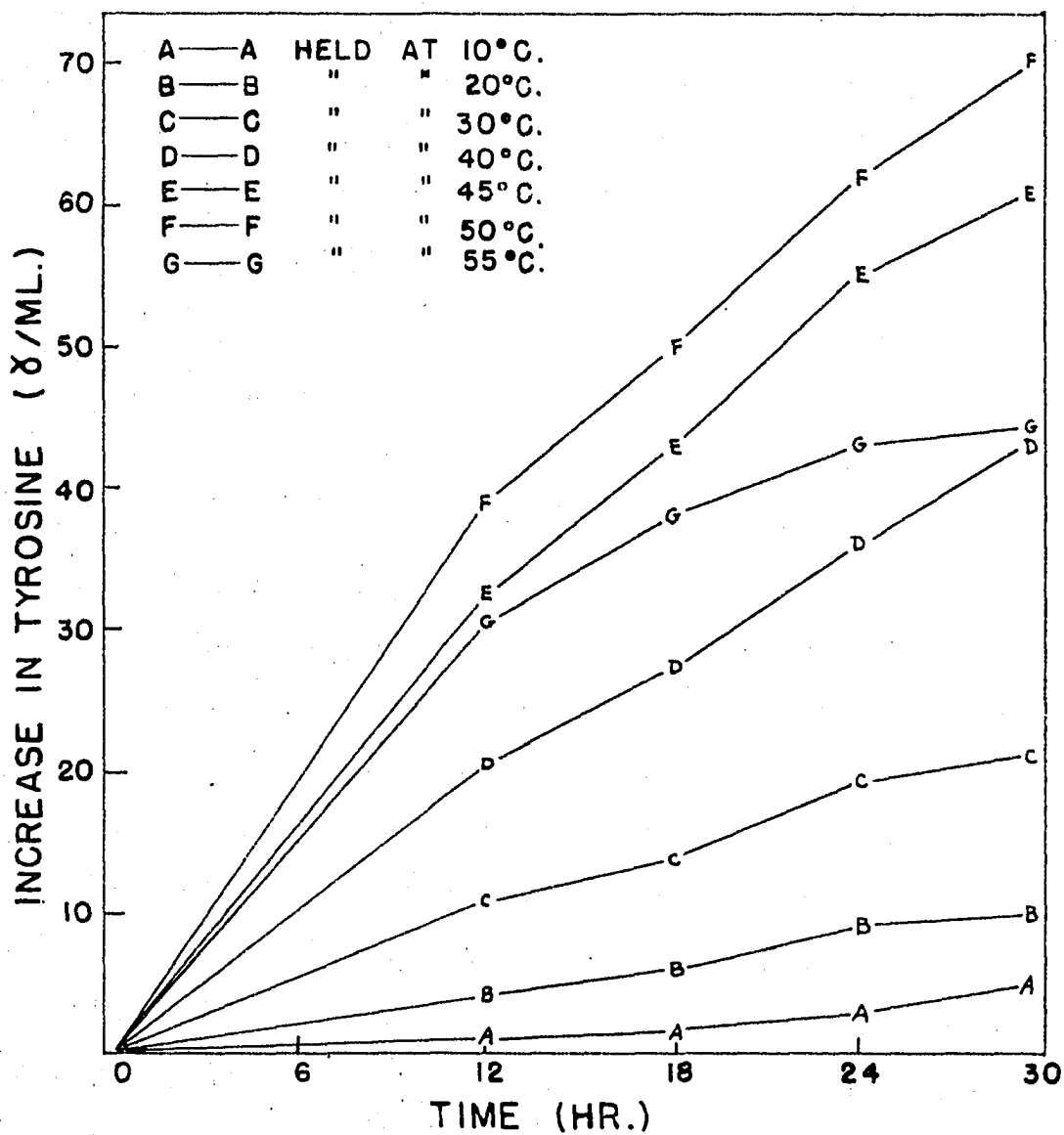


Figure 7. Effect of time and temperature on proteinase activity by cell-free extract of *L. casei* tested on 2 per cent casein suspension at pH 6.0

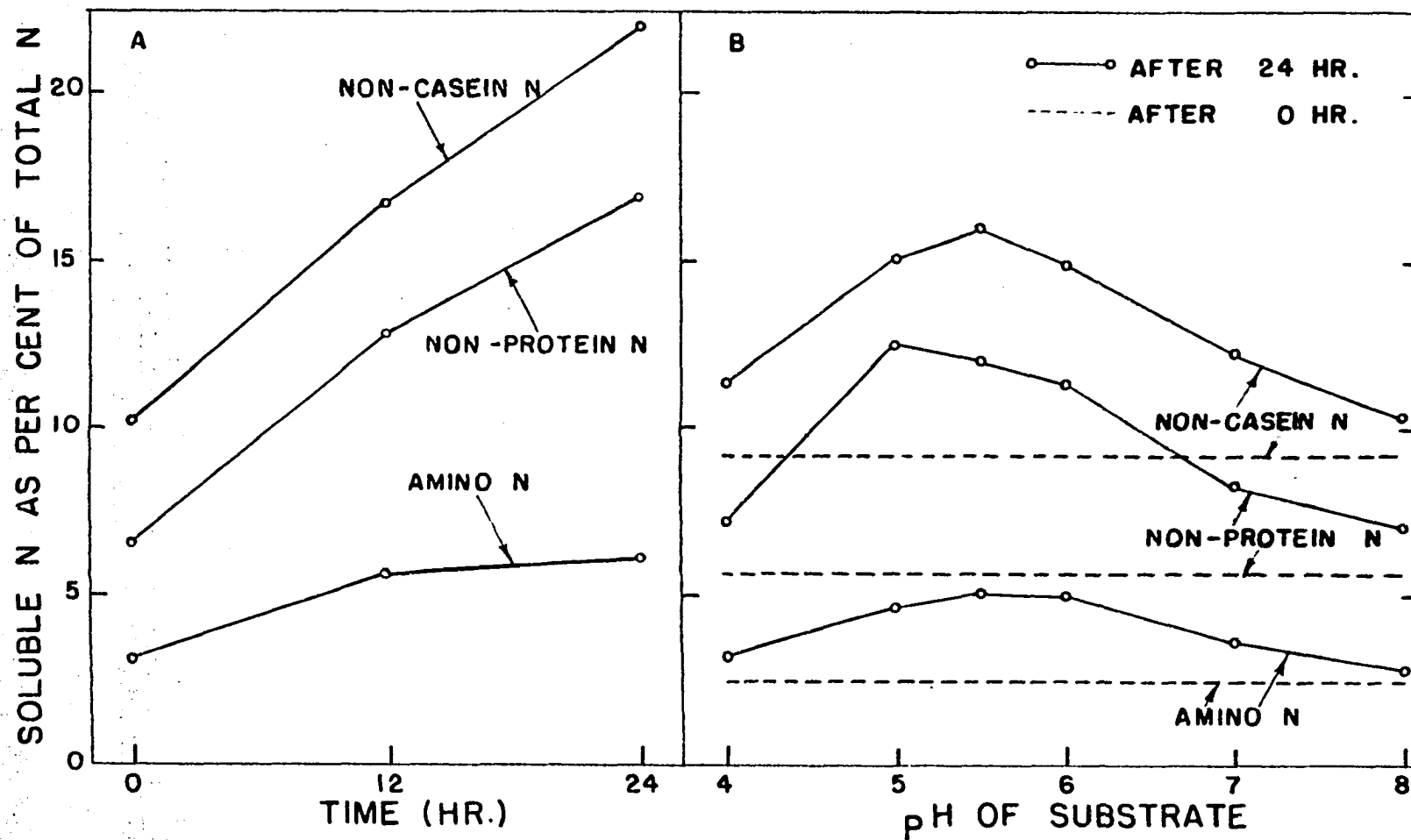


Figure 8. Effect of time and pH on the increase in non-casein, non-protein and amino nitrogen. A = 180 ml. of 2 per cent casein suspension and 20 ml. cell-free extract, pH 6.0, incubated at 45°C. B = 50 ml. of 2 per cent casein suspension and 3 ml. cell-free extract incubated at 45°C. for 24 hours

very closely. The increase in non-casein and non-protein nitrogen followed mainly the same pattern. In both cases the rate of formation of the respective breakdown products slowed down somewhat after 12 hours. The initial rate of increase in amino nitrogen was considerably less than for the two other fractions. The decrease in rate of formation of amino nitrogen after 12 hours was approximately the same or possibly slightly greater than in the case of non-casein and non-protein nitrogen.

Effect of pH on the increase in non-casein, non-protein and amino nitrogen by cell-free extract of *L. casei*

The digestion mixtures consisted of 50 ml. of 2 per cent casein suspension and 3 ml. of cell-free extract in 125 ml. erlenmeyer flasks. The reaction of the mixtures was adjusted to desired pH with 5 N sodium hydroxide or 5 N sulfuric acid. The flasks were incubated at 45°C. for 24 hours. Tests for total, non-casein, non-protein and amino nitrogen were run on 10 ml. samples at the beginning and at the end of the incubation period. The results shown in Figure 8B are given as nitrogen in per cent of total nitrogen, and are based on duplicate runs.

According to the results obtained, the maximum increase in the different fractions occurred at slightly different pH levels. The increases in non-casein and amino nitrogen were maximum at pH 5.5. The maximum increase in non-protein nitrogen occurred closer to pH 5.0. Fifty per cent of maximum increase in non-casein nitrogen and non-protein nitrogen occurred at approximately pH's 4.5 and 6.5. The corresponding values for amino nitrogen were approximately pH's 4.5 and 7.0, respectively.

Effect of heating at different pH levels on proteinase activity by cell-free extract of *L. casei*

Cell-free extract in 10 ml. portions was adjusted to desired pH with 5 N sodium hydroxide or 5 N sulfuric acid. The volumes were made up to 12 ml., and then distributed in 3 ml. portions into screw-cap tubes. For each pH, one tube was not heated and three tubes were heated to 61.7°C. for 2, 5 and 10 minutes, respectively, and immediately cooled in ice water. The effect of the heat treatment was tested on 2 per cent casein suspension. The digestion mixture consisted of 5 ml. of casein suspension, 1 ml. of cell-free extract and 1 ml. of sterile distilled water, all at pH 6.0. Incubation was at 45°C. for 24 hours. Proteinase activity was determined by the colorimetric method for tyrosine and tryptophane, and the results are shown in Table 5.

Table 5. Effect of heating to 61.7°C. at various pH levels on proteinase activity by cell-free extract of *L. casei*, as measured by the increase in non-protein nitrogen

pH during heating	Increase in tyrosine (γ /ml. substrate) when enzyme was heated for:			
	0 min.	2 min.	5 min.	10 min.
5	98	89	73	58
6	98	83	65	51
7	98	35	24	17
8	98	19	15	3
9	98	10	8	0

The stability of the enzyme at 61.7°C. was found to be highest at pH 5.0. The stability was only slightly less at pH 6.0, but decreased rapidly as the pH was increased to 7.0 or above. The enzyme retained 60 per cent of its activity after heating at pH 5.0 to 61.7°C. for 10 minutes, but was completely inactivated at pH 9.0 after the same length of time.

A similar experiment was run a second time. The reaction of the heated enzyme varied from pH 4.5 to 7.0, at one-half pH unit intervals. The temperature and times of heating were the same as before. The activity of the heated enzyme was determined by increase in non-casein nitrogen in a digestion mixture consisting of 10 ml. of 2 per cent casein suspension and 1.5 ml. of cell-free extract, pH 6.0. The mixture was incubated at 45°C. for 24 hours. Non-casein nitrogen in the acetate filtrate was determined by Kjeldahl's method. The results shown in Table 6 are the average values from duplicate runs which agreed closely.

Table 6. Effect of heating to 61.7°C. at various pH levels on proteinase activity by cell-free extract of *L. casei*, as measured by the increase in non-casein nitrogen

pH during heating	Per cent of total nitrogen converted to non-casein nitrogen by enzyme heated for:			
	0 min.	2 min.	5 min.	10 min.
4.5	11.6	6.2	3.6	2.8
5.0	11.6	10.2	7.1	5.2
5.5	11.6	10.0	7.3	5.0
6.0	11.6	7.1	4.3	2.4
6.5	11.6	3.1	1.4	0.5
7.0	11.6	1.2	0.2	0

The minimum inactivation of the proteinase by heating to 61.7°C. was found to occur at pH 5.0 to 5.5. Enzyme heated at pH 4.5 retained slightly more than half of the activity retained after heating at pH 5.0. The heating stability of the enzyme at pH 6.0 was slightly higher than at pH 4.5 when heated for 2 or 5 minutes. After 10 minutes of heating to 61.7°C., more activity was retained at pH 4.5 than at pH 6.0. In comparing this run with the previous one (Table 5), it is seen that less heat stability at the higher pH levels was obtained in the last run, whereas the stability at pH 5.0 was approximately the same in both cases. The reason for the variations may be that there are two different enzyme systems involved; it may also be due to differences in the protein concentration in the two cell-free extracts used in these experiments.

Peptidase Activity by Cell-free Extracts of L. casei 25

Effect of temperature on peptidase activity by cell-free extract of L. casei

The effect of temperature on peptidase activity by the cell-free extract was tested on M/15 DL-leucylglycine and DL -alanyglycine and M/30 glycyL-tyrosine and glycyL-leucine. The digestion mixture consisted of 2.5 ml. substrate at pH 7.0 and 0.2 ml. of cell-free extract in screw-cap tubes. The tubes were incubated in a water bath at the desired temperatures for 1 hour. Per cent hydrolysis was determined by titration at the beginning and at the end of the incubation period, as

described in section Materials and Methods. The obtained results are shown in Figure 9.

The optimum temperature for peptidase activity was found to be 45 to 50°C., and there was little difference in temperature optima for hydrolysis of the four dipeptides tested. The decrease in rate of hydrolysis at temperatures above 50°C. was less pronounced than it was in case of the proteinase activity.

Effect of pH on peptidase activity by cell-free extract of *L. casei*

The effect of pH on the peptidase activity was tested on seven dipeptides and two tripeptides. The substrates were adjusted to desired pH with 1 N sodium hydroxide or 1 N hydrochloric acid. The digestion mixture consisted of 2.5 ml. of substrate and 0.2 ml. of cell-free extract. Tests were run at 45°C. for 1 hour, and per cent hydrolysis was determined by titration of 1 ml. of the digestion mixture at the beginning and at the end of the incubation period, as described under Materials and Methods. The results shown in Figure 10 were, within the limits of a low-level of experimental error, reproducible using cell-free extracts obtained from different batches of *L. casei*.

The pH optima for hydrolysis of the peptides tested were all within the range of pH 7.0 to 8.0. Generally it was found that the pH optima for hydrolysis of the glycyl-peptides occurred at a higher pH level than was the case for the hydrolysis of the alanyl- and leucyl-peptides. DL-alanyl-DL-alanine was rapidly hydrolyzed over a wide pH range. Glycylglycine and diglycylglycine were hydrolyzed rather slowly, but

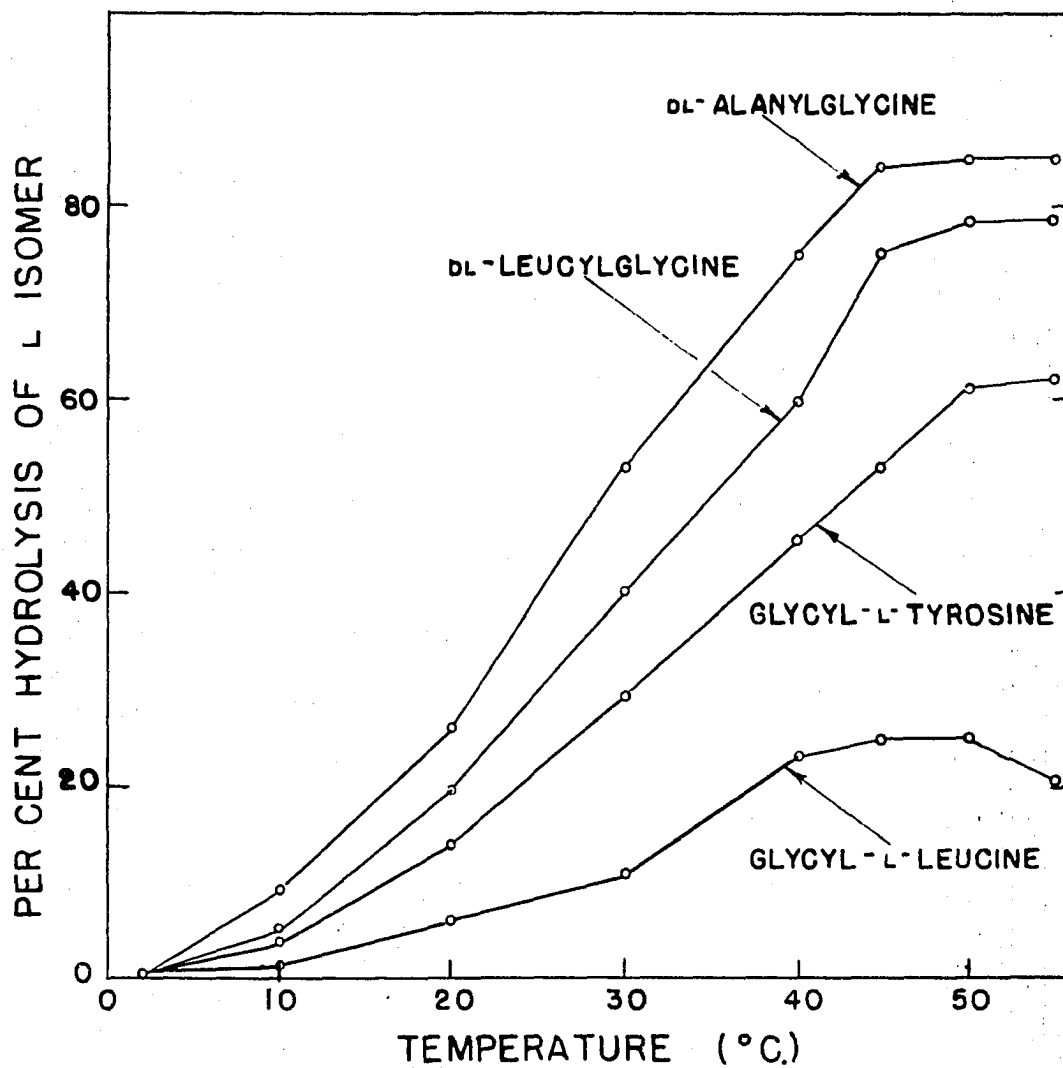


Figure 9. Effect of temperature on peptidase activity at pH 7.0 by cell-free extract of *L. casei* (0.2 ml. cell-free extract in 2.5 ml. substrate. Incubated for 1 hour)

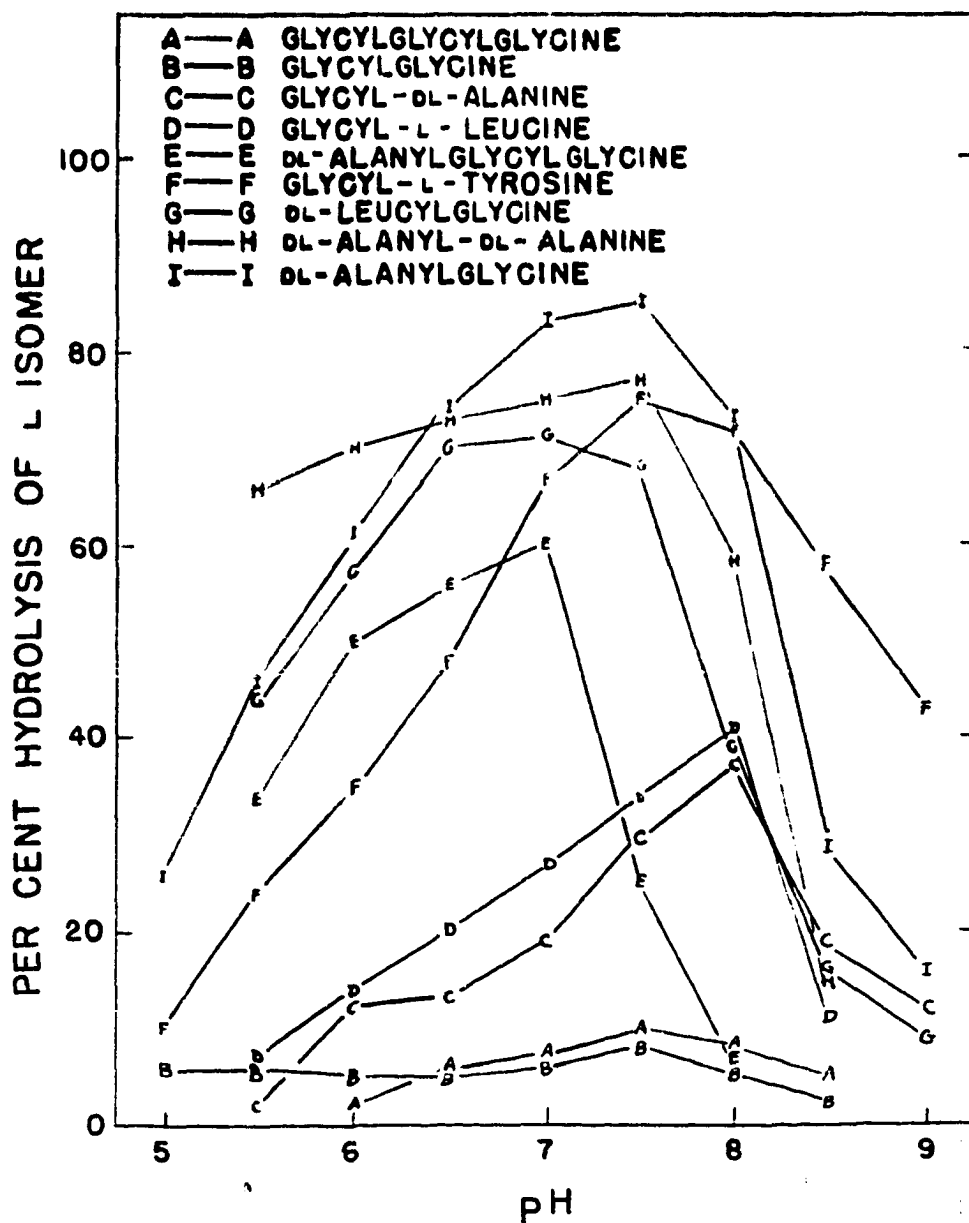


Figure 10. Effect of pH on peptidase activity by cell-free extract of *L. casei*. (0.2 ml. cell-free extract in 2.5 ml. substrate, Incubated at 45°C. for 1 hour)

over a wide pH range. DL-alanylglycylglycine was hydrolyzed rapidly between pH 6.0 and 7.0, but a sharp decline in rate of hydrolysis was observed at the higher pH levels. DL-alanylglycine was hydrolyzed at the highest rate, but DL-leucylglycine, glycyl-L-tyrosine and DL-alanyl-DL-alanine also were hydrolyzed rapidly. Glycyl-L-leucine and glycyl-DL-alanine showed similarities as to rate of hydrolysis and pH optima. There was some indication of two pH optima for hydrolysis of glycyl-DL-alanine.

Effect of metallic ions on peptidase activity by cell-free extract of L. casei

The effect of metallic ions on the peptidase activity of cell-free extract of L. casei was tested on eight dipeptides, three tripeptides and on carbobenzoxyglycyl-L-phenylalanine. The following metal salt solutions in 0.05 M concentrations were used: $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{CuSO}_4 \cdot 4\text{H}_2\text{O}$, $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Two tenths of a ml. of the desired salt solution was incubated with 1.8 ml. of cell-free extract at 45°C . for 15 minutes, for the control 0.2 ml. of distilled water was used instead of the metal salt solution. The ion-treated cell-free extract in 0.2 ml. quantity was added to 2.5 ml. of substrate, giving a concentration of metallic ions in the digestion mixture of approximately 0.0004 M. The tests were run at 45°C . for 1 hour, and the results obtained are shown in Table 7.

Cobalt had the greatest effect on the enzymatic hydrolysis of the peptides, and this effect was to some degree dependent on the pH of the substrate. The hydrolysis of glycyl-DL-alanine and glycyl-L-leucine

Table 7. Effect of metallic ions on peptidase activity by cell-free extract of L. casei^a

Substrate	pH	Per cent hydrolysis in presence of the following ions ^b						
		None	Co ⁺⁺	Zn ⁺⁺	Ni ⁺⁺	Cu ⁺⁺	Mn ⁺⁺	Mg ⁺⁺
Glycyl-DL-alanine	6.0	11	72	11	16	6	11	10
Glycyl-DL-alanine	8.0	24	37	46	38	30	23	30
Glycyl-L-leucine	6.0	13	61	12	-	-	-	-
Glycyl-L-leucine	7.7	44	62	43	45	45	40	46
Glycyl-L-tyrosine	8.0	68	53	85	58	62	62	64
Glycyl-L-tryptophane	7.0	6	36	-	-	-	-	-
Glycylglycine	7.7	8	32	11	11	3	8	2
Diglycylglycine	7.0	6	2	6	6	-	6	2
DL-alanylglycine	7.0	91	35	88	54	77	70	80
DL-leucylglycine	6.0	40	73	-	-	-	-	-
DL-leucylglycine	7.0	82	25	83	23	27	29	83
DL-alanyl-DL-alanine	6.0	36	35	35	37	32	37	35
DL-alanyl-DL-alanine	7.0	35	32	37	33	35	40	35
DL-alanylglycylglycine	7.0	52	17	66	3	11	11	56
DL-leucylglycylglycine	7.0	42	14	50	-	-	-	-
Cbo. ^c glycyl-L-phenylalanine	6.0	0	0	-	-	-	-	-
Cbo. glycyl-L-phenylalanine	7.5	0	0	-	-	-	-	-

^a0.2 ml. of cell-free extract in 2.5 ml. of substrate, incubated at 45°C. for 1 hour.

^bIon concentration in digestion mixture was 0.0004 M.

^cCbo. = Carbobenzoxy.

was greatly activated by cobalt at pH 6.0, but activated considerably less at pH 8.0 and 7.7, respectively. Cobalt also activated the hydrolysis of glycylglycine at pH 7.7 and glycyl-L-tryptophane at pH 7.0. The hydrolysis of glycyl-L-tyrosine at pH 8.0 and of DL-alanylglycine, DL-leucylglycine, diglycylglycine, DL-alanylglycylglycine and DL-leucylglycylglycine at pH 7.0 was inhibited by cobalt ions. DL-alanyl-DL-alanine was not affected by cobalt at either pH 6.0 or 7.0. Carbo-benzoxylglycyl-L-phenylalanine at pH's 6.0 and 7.5 was not hydrolyzed by the cell-free extract with or without added cobalt ions. Zinc activated the hydrolysis of glycyl-DL-alanine at pH 8.0 and also slightly activated the hydrolysis of glycyl-L-tyrosine at pH 8.0 and DL-alanylglycylglycine and DL-leucylglycylglycine at pH 7.0. Otherwise it did not have any significant effect on the hydrolysis of the peptides tested. Nickel activated the hydrolysis of glycyl-DL-alanine at pH 6.0 and 8.0 and strongly inhibited the hydrolysis of DL-alanylglycine, DL-leucylglycine and DL-alanylglycylglycine at pH 7.0, but had little effect on the hydrolysis of the remaining peptides. Copper and manganese were inhibitory or had no effect on the peptide hydrolysis. Magnesium inhibited the hydrolysis of glycylglycine and diglycylglycine, but otherwise had little effect on the enzymatic hydrolysis of the rest of the peptides tested. It was interesting to note that whereas the hydrolysis of DL-leucylglycine was strongly inhibited by nickel, copper and manganese, the same ions only slightly inhibited the hydrolysis of DL-alanylglycine. Cobalt in both cases strongly inhibited the hydrolysis.

A test was run in order to establish whether it was necessary to add the ions to the cell-free extract or whether the same results would be obtained by adding the ions to the substrates. In adding the ions to the cell-free extract, the same method was used as previously described. In the other method, 0.04 ml. of the 0.05 M metal salt solution was added to 4.96 ml. of substrate. In both cases 2.5 ml. of substrate and 0.2 ml. of cell-free extract were used in the digestion mixture, which was incubated at 45°C. for 1 hour. In Table 8 are shown the results obtained when cobalt was used as an activator in the enzymatic hydrolysis of glycy-DL-alanine at pH 6.0.

Table 8. Activation of the hydrolysis of glycy-DL-alanine by cobalt when added to the cell-free extract and when added to the substrate

Method	Per cent hydrolysis
No ions added	10
Ions added to cell-free extract	61
Ions added to substrate	57

The results are given as the average of duplicate runs.

On the basis of these results there did not seem to be any significant difference between the two methods. This was further established by additional runs, and during the remainder of this work the metal salt solutions have been added to the substrates, since it was found to be more convenient.

Effect of pH on metal ion activation. The results in Table 7 indicated that the effect of metallic ions on the enzymatic hydrolysis of peptides might be affected by variations in pH. It therefore was decided to test the effect of cobalt and zinc ions on the hydrolysis of glycyl-DL-alanine, DL-alanyl-glycine, glycyl-L-tyrosine and glycylglycine at various pH levels. The substrates, in 15 ml. samples, were adjusted to the desired pH and divided into 5 ml. samples. Metal salt solutions were added to give a metal ion concentration of 0.0004 M. Equivalent amounts of water were added to the controls. The digestion mixture consisting of 2.5 ml. of substrate and 0.2 ml. of cell-free extract was incubated at 45°C. for 1 hour. The results shown in Figures 11 and 12 were confirmed in additional runs.

Cobalt in all cases activated the hydrolysis at the lower pH levels, resulting in maximum rate of hydrolysis at pH 5.5. The hydrolysis of glycyl-DL-alanine (Figure 12A), glycyl-L-tyrosine (Figure 11A) and glycylglycine (Figure 11B) in the presence of cobalt had a second rate optimum at pH 7.5 or higher. Hydrolysis of glycyl-L-tyrosine was slightly inhibited by cobalt at the higher pH levels, but the hydrolysis of glycylglycine was strongly activated by cobalt over a wide pH range. The rate of hydrolysis of glycyl-L-tyrosine and glycylglycine in the presence of cobalt was similarly affected by variations in pH. In the case of glycyl-DL-alanine, there was a much higher rate of hydrolysis at the lower pH levels than at the higher pH levels when cobalt was present. The rate of hydrolysis of DL-alanyl-glycine in the presence of cobalt was maximum at pH 5.5 (Figure 12B), but it differed from the three

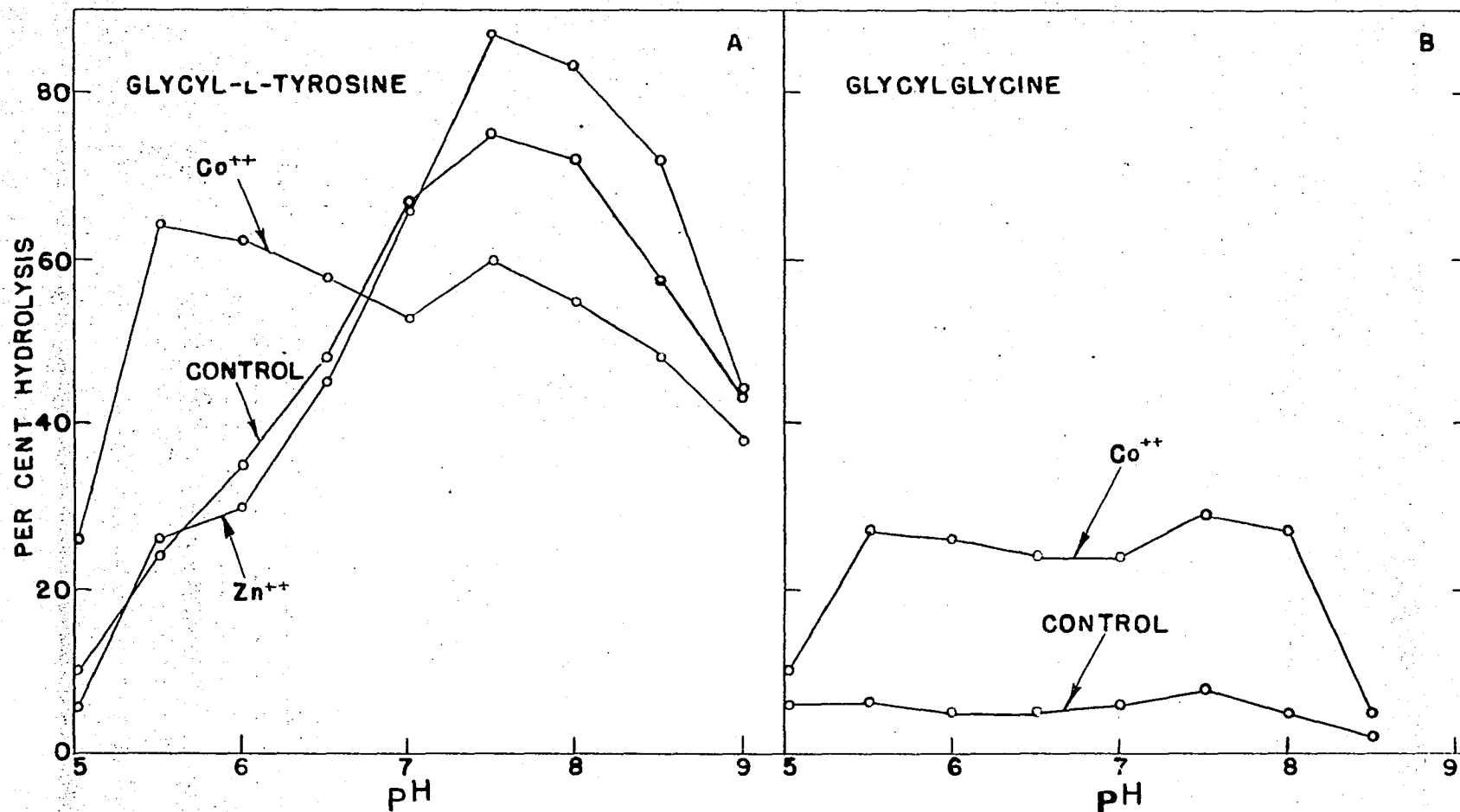


Figure 11. Effect of cobalt and zinc at various pH levels on the enzymatic hydrolysis of peptides. (0.2 ml. cell-free extract in 2.5 ml. substrate containing 0.0004 M concentration of metal ions. Incubated at 45° C. for 1 hour)

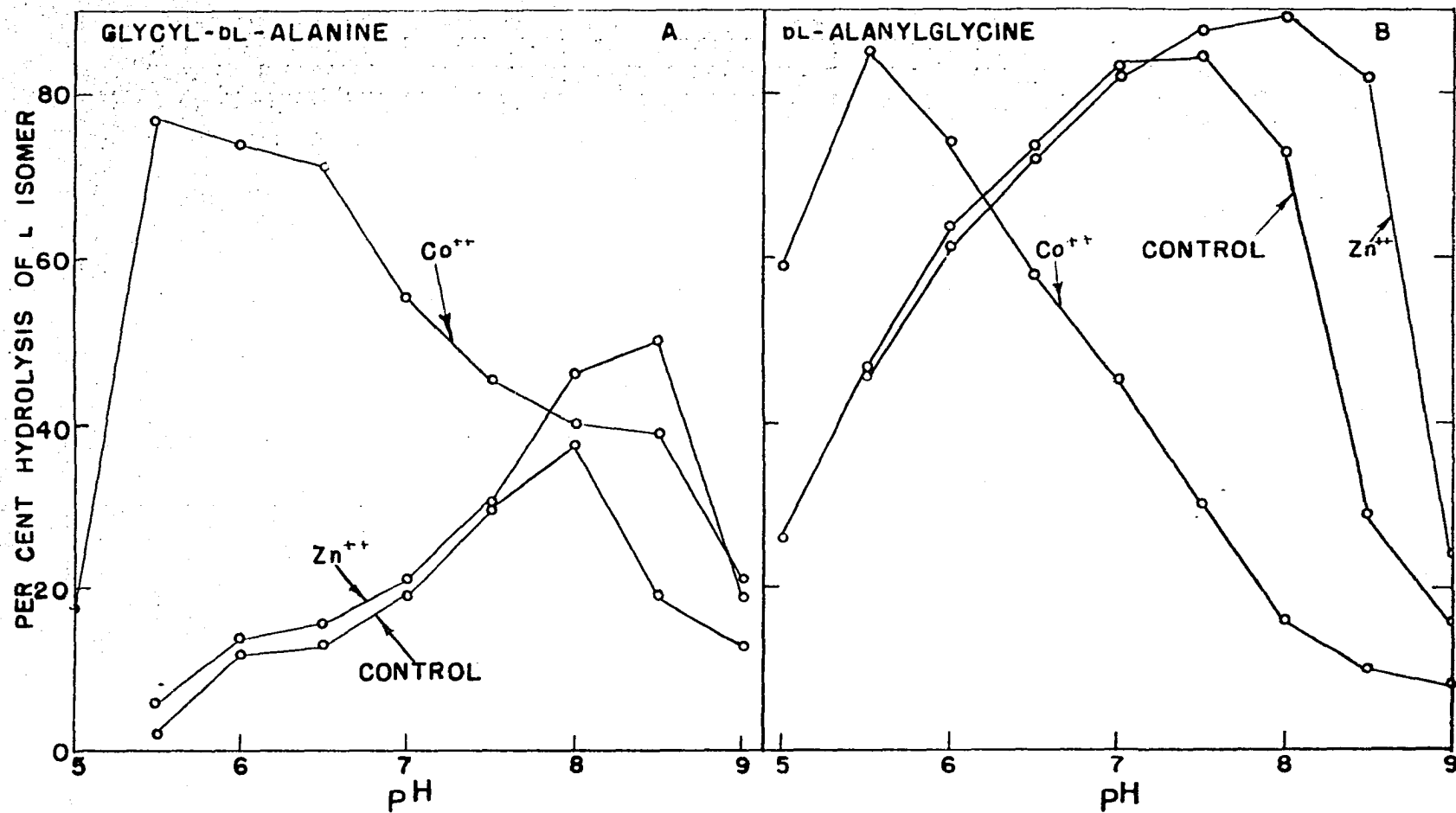


Figure 12. Effect of cobalt and zinc on the enzymatic hydrolysis of peptides at various pH levels. (0.2 ml. cell-free extract in 2.5 ml. substrate containing 0.0004 M concentration of metal ions. Incubated at 45° C. for 1 hour)

glycylpeptides tested in that a second rate optimum for hydrolysis was not observed. The rate of hydrolysis of DL-alanylglycine decreased rapidly with increasing pH when cobalt was present, and was strongly inhibited by cobalt at pH 6.5 and above.

Zinc did not have any effect on the hydrolysis of the peptides at pH 7.0 or below, but the hydrolysis of glycyl-L-tyrosine, glycyl-DL-alanine and DL-alanylglycine was activated by zinc at the higher pH levels.

Effect of metal ion concentration. The effect of various concentrations of cobalt on the enzymatic hydrolysis of DL-alanylglycine and glycyl-L-tyrosine at different pH levels was tested. The concentrations of cobalt in the digestion mixture were 0.0004, 0.00008 and 0.000008 M. The digestion mixture otherwise consisted of 2.5 ml. of substrate of the desired pH and 0.2 ml. of cell-free extract. Incubation was at 45°C. for 1 hour. The results obtained are shown in Figure 13.

The activation by cobalt at pH 5.5 was found to decrease with decreasing metal ion concentration, and in case of the lowest concentration used, the hydrolysis of DL-alanylglycine (Figure 13A) and glycyl-L-tyrosine (Figure 13B) was actually inhibited at pH 5.5. The hydrolysis of DL-alanylglycine at reactions of pH 6.5 or above, and the hydrolysis of glycyl-L-tyrosine at reactions of pH 7.0 or above were inhibited by cobalt in all three cobalt concentrations used.

Effect of cobalt and zinc on the hydrolysis of DL-alanylglycine. An experiment was carried out adding both cobalt and zinc ions to the digestion mixture of DL-alanylglycine and cell-free extract. The digestion was carried out at 45°C. for 1 hour and at pH's 5.5 and 7.0. Controls were

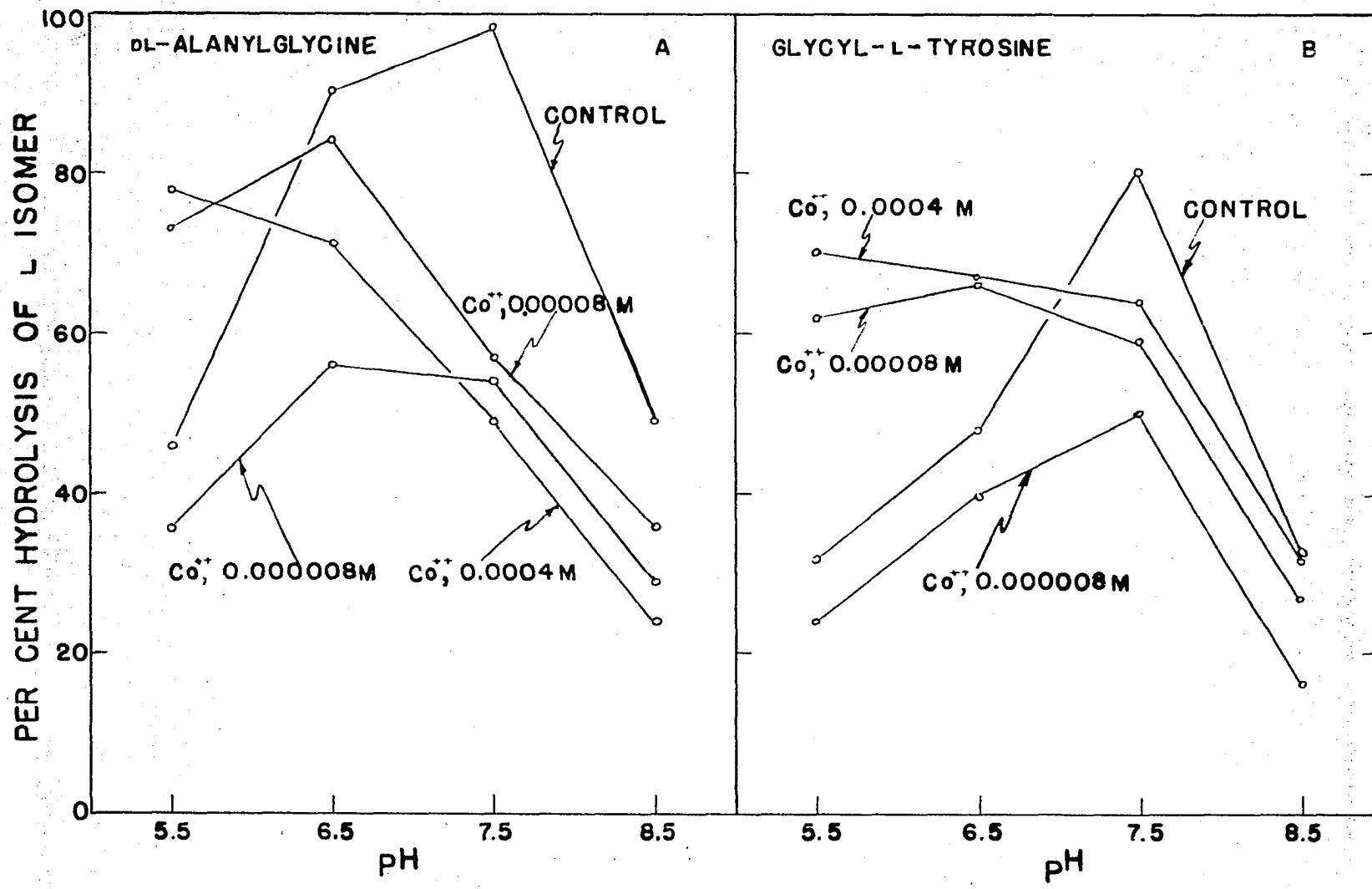


Figure 13. Effect of various concentrations of cobalt on the enzymatic hydrolysis of peptides at different pH levels. (0.2 ml. cell-free extract in 2.5 ml. substrate. Incubated at 45°C. for 1 hour)

run with no ions added and with addition of cobalt alone and zinc alone. The concentration of cobalt was in all cases 0.0025 M. In two of the digestion mixtures containing cobalt, zinc was added at a concentration of 0.001 M and 0.0025 M, respectively. The results shown in Table 9 are average values of duplicate runs. The effect of cobalt was partly eliminated in presence of 0.001 M concentration of zinc. Where the concentrations of cobalt and zinc ions were equal, the effect of cobalt was eliminated completely.

Table 9. Effect of Co^{++} and Zn^{++} on hydrolysis of DL-alanylglycine

pH	Per cent hydrolysis in presence of:				
	No added ions	Co^{++} 0.0025 <u>M</u>	Zn^{++} 0.0025 <u>M</u>	Co^{++} 0.0025 <u>M</u> + Zn^{++} 0.0010 <u>M</u>	Co^{++} 0.0025 <u>M</u> + Zn^{++} 0.0025 <u>M</u>
5.5	62	85	62	67	61
7.0	93	51	95	91	96

Effect of heating at different pH levels on peptidase activity by cell-free extract of L. casei

The experiment was carried out as described for the corresponding experiment on the proteinase activity, with the exception that the cell-free extract was heated to 61.7°C. for 2 minutes only. The heated enzyme was then tested on a series of peptides. Controls were run with unheated enzyme. The results in Figure 14 were closely reproduced in a second run.

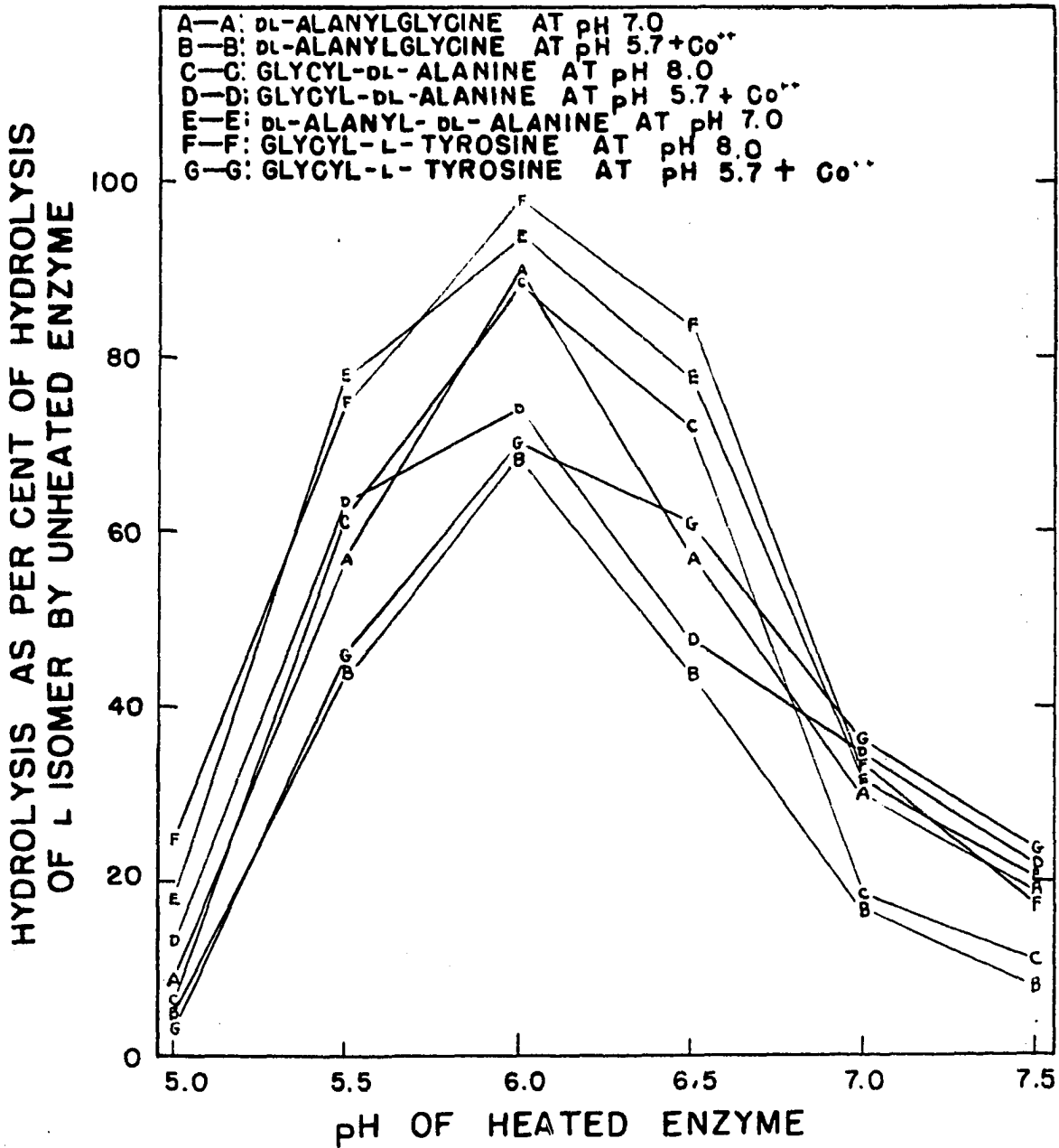


Figure 14. Effect of heating to 61.7°C. at different pH levels on peptidase activity by cell-free extract of *L. casei*

Maximum stability of the peptidase activity occurred at pH 6.0. The enzyme was rapidly heat inactivated below pH 5.5 and above pH 6.5. The enzyme activities tested in presence of cobalt were less stable than the activities in absence of cobalt. Enzyme heated to 61.7°C. at pH 6.0 for 2 minutes retained 88 per cent or more of its activity when tested on DL-alanylglycine and DL-alanyl-DL-alanine at pH 7.0 and glycyl-DL-alanine and glycyl-L-tyrosine at pH 8.0 and in the absence of cobalt. Less than 75 per cent of the peptidase activity on three of the same peptides tested at pH 5.7 in presence of cobalt was retained under the same conditions.

Purification of the Cell-free Extract

Purification by fractional precipitation and adsorption

Purification of the cell-free extract and separation of the various proteolytic enzyme systems present were attempted. During the course of this procedure, fractional precipitation with ammonium sulfate was used. In order to determine the concentrations of ammonium sulfate to be used for maximum recovery of the enzyme with minimum amount of inert proteins, tests were run with different concentrations of ammonium sulfate. The tests were run on 4 ml. samples of cell-free extract in which the desired degree of saturation was obtained by adding dry ammonium sulfate. The amounts needed could be calculated on the basis of data given in specially prepared tables (24). The ammonium sulfate was added carefully under stirring and in the cold. Precipitation was allowed to take

place for 1 hour. The precipitate was centrifuged out and the supernatant discarded. The precipitate was suspended in 0.03 M phosphate buffer at pH 7.0 and made up to 4 ml. Recovery of the peptidase activities in the various fractions was tested on glycylglycine, DL-alanylglycine, glycyl-DL-alanine and glycyl-L-tyrosine. Recovery of the proteinase activity was tested on 2 per cent casein suspension at pH 6.0, and was measured by the colorimetric method for tyrosine and tryptophane. The results obtained are shown in Figure 15.

The major part of the peptidase activity was recovered in the fraction not precipitated at 0.45 saturation with ammonium sulfate, but precipitated at 0.7 saturation. Most of the proteinase activity also was recovered in this fraction. The fact that more than 100 per cent recovery apparently was obtained, may be due to removal of some inert proteins which could have slowed down the peptidase activity in the crude extract. The break in the curve for recovery of proteinase activity may indicate the presence of two proteinase systems in the cell-free extract.

The procedure used in purification of the cell-free extract is outlined in Figure 16. All the work was carried out at 2°C. The first step was fractional precipitation with ammonium sulfate. Dry ammonium sulfate was added carefully under mechanical stirring, and precipitation was allowed to take place for 1 hour before centrifugation. The fraction soluble at 0.4 saturation with ammonium sulfate, but not soluble at 0.75 saturation, contained most of the proteolytic activity, and was used for further purification. This fraction was suspended in minimum amount of 0.03 M phosphate buffer at pH 7.0, and dialyzed for 10 hours against 18 l. of distilled water.

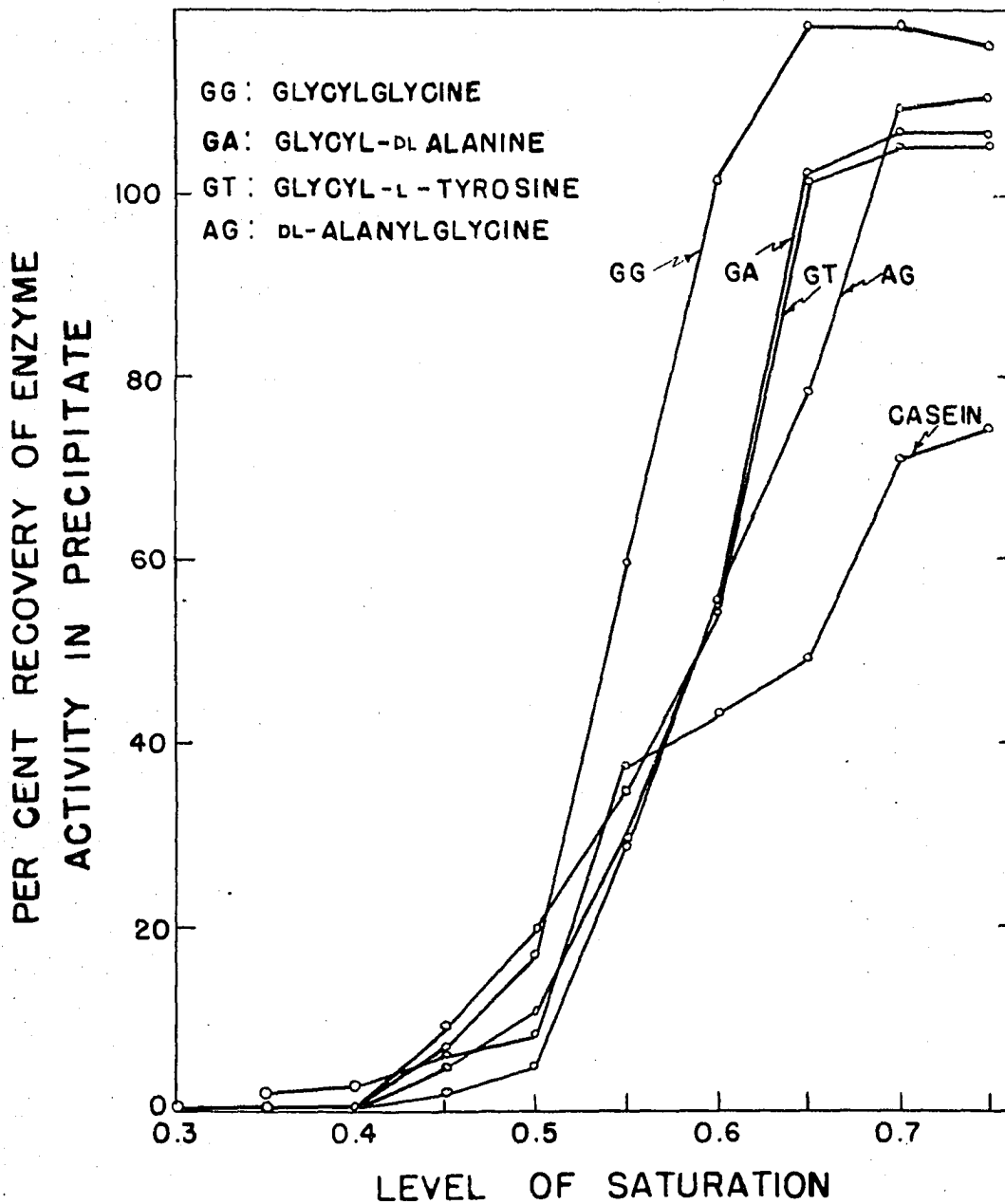
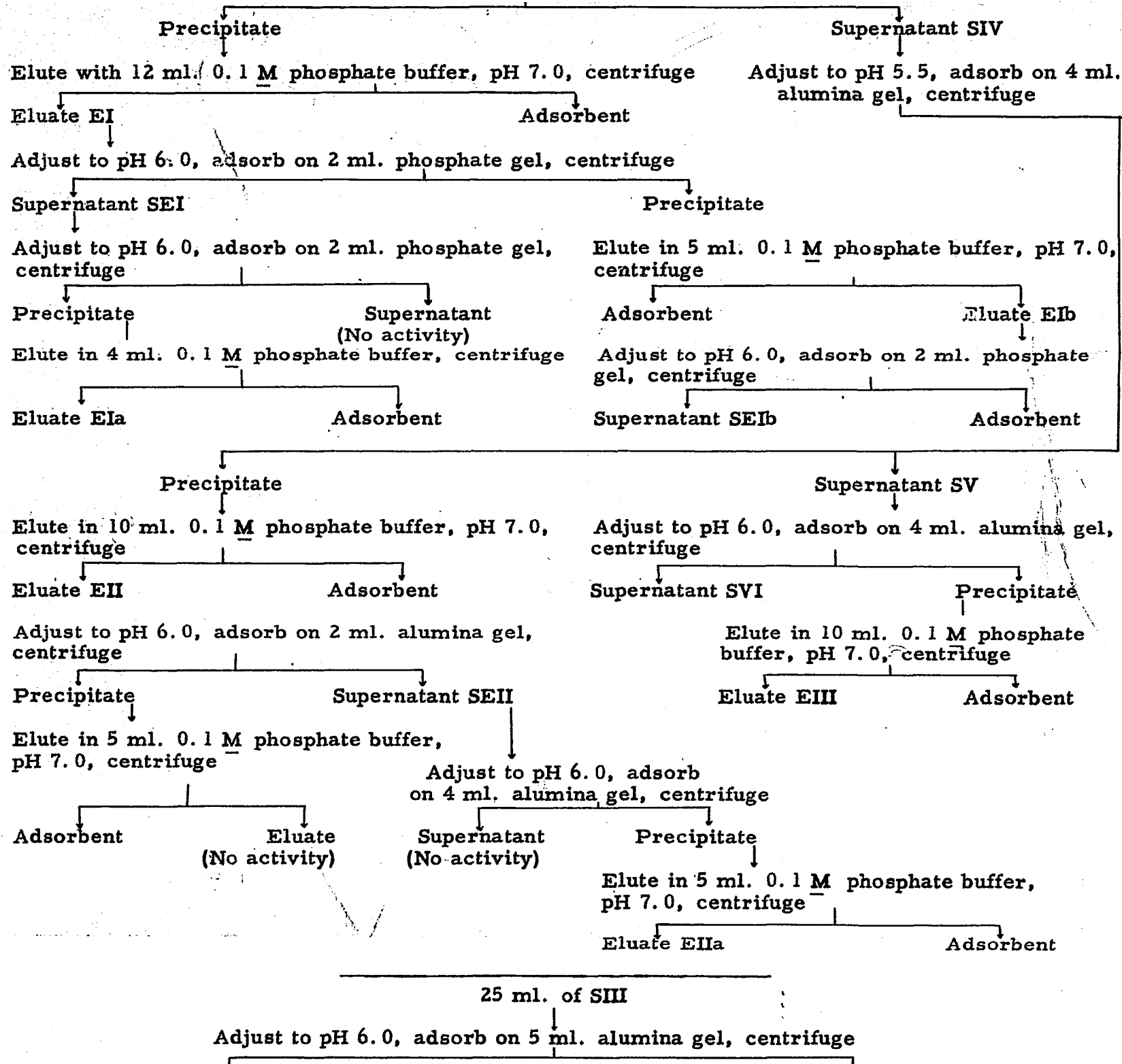
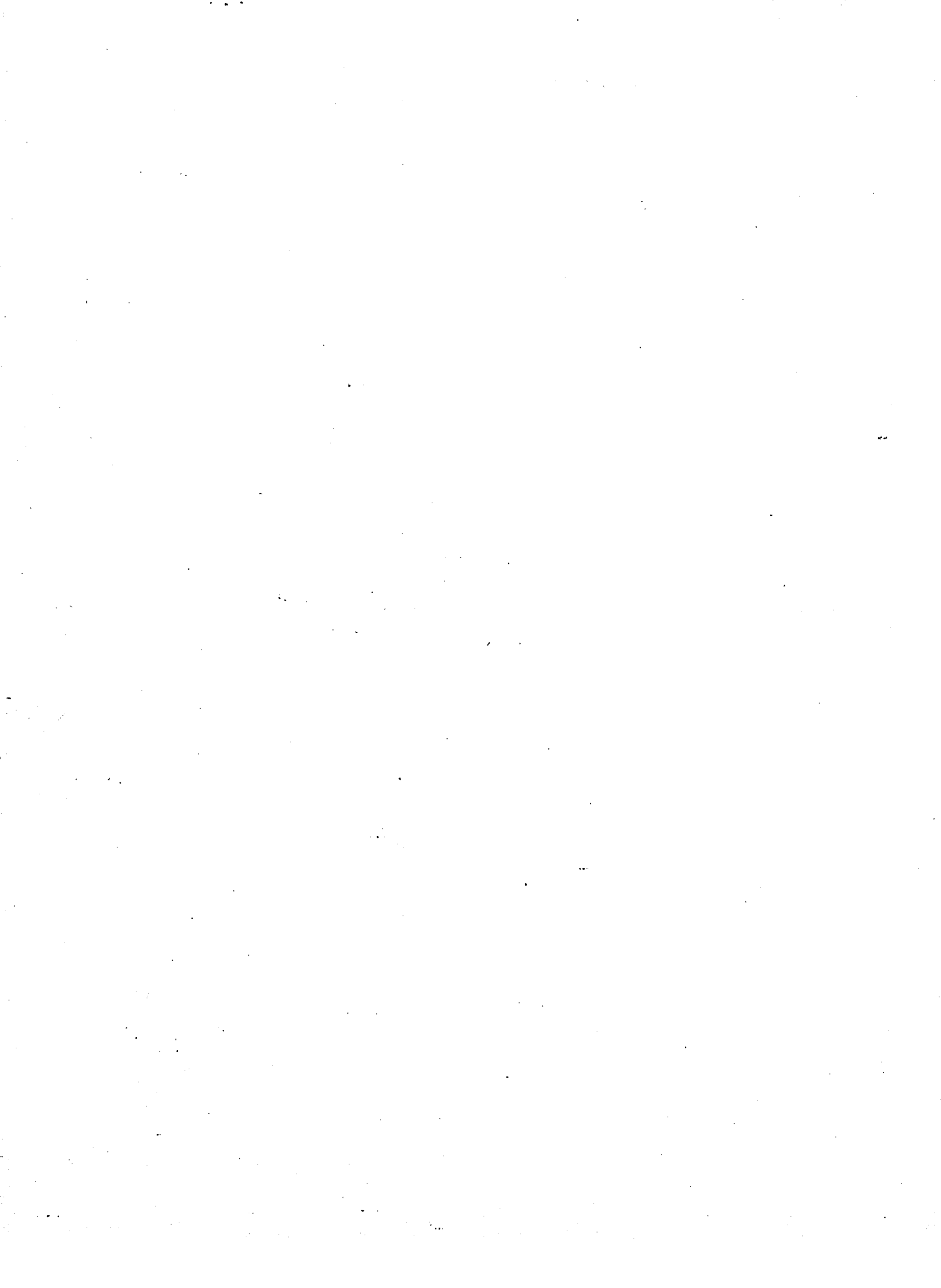


Figure 15. Recovery of enzyme activity in fraction of cell-free extract precipitated with $(\text{NH}_4)_2\text{SO}_4$ at various levels of saturation. Peptidase activity tested on four peptides, and proteinase activity tested on a 2 per cent casein suspension









For removal of nucleic acids, the reaction was adjusted to pH 5.5, and a protamine solution (20 mg./ml., pH 5.5) was added carefully under continuous stirring. After 20 minutes the precipitate was removed by centrifugation. The amount of protamine solution added depended somewhat on the protein concentration in the cell-free extract. Enough protamine was used to bring the ratio of light absorption of the enzyme suspension at 280 $m\mu$ to 260 $m\mu$ from an approximate initial value of 0.5 up to 1.0. The supernatant (SIII), after the protamine precipitation, then was purified by selective adsorption and elution as indicated in Figure 16. The adsorbents were added carefully under stirring, and all adsorptions and elutions were allowed to take place for 20 minutes. The enzymatic activities and the protein contents of the various fractions are shown in Table 10. A considerable degree of purification was obtained by the fractional precipitation and adsorption. The precipitation of nucleic acids with protamine turned out to be a very effective way of purifying the peptidase activity. More than 75 per cent of the proteinaceous material was removed by this method, whereas more than 75 per cent of the peptidase activity remained in the purified fraction (SIII). It was interesting to note that all the proteinase activity could be removed by this protamine precipitation, whereas most of the peptidase activity remained in solution. All the proteinase activity could not be recovered from the precipitate by the method used here. In washing the precipitate with 0.03 M phosphate buffer, part of the proteinase activity, together with some peptidase activity, was recovered. The peptidase activity was highest on

Table 10. Proteolytic activity of cell-free ext

Fraction tested	Protein nitrogen (mg./ml.)	Per cent hydrolysis of L-isomer of t				
		AG pH 7.0	AG pH 5.7 +CO ⁺⁺ c	GA pH 8.0	GA pH 5.7 +CO ⁺⁺	GT pH 8.0
Cell-free extract	4.60	90	75	29	53	59
DP II	3.80	86	75	25	48	53
SP III	2.40	24	23	0	3	-
SP IIIa	1.45	0	0	-	-	-
S III	0.90	66	64	16	37	62
E I	0.80	91	91	43	61	64
E Ia	0.10	45	-	11	-	-
E Ib	0.08	59	70	19	32	51
SE Ib	0.08	50	58	6	26	18
E II	0.90	100	96	27	88	-
E IIa	0.10	40	43	0	21	20
E III	0.05	23	24	0	13	-
E IVa	0.10	85	90	24	61	-
E IVb	0.12	61	-	8	-	-
E IVc	0.28	31	32	0	18	11

^a 0.2 ml. enzyme in 2.5 ml. substrate (peptide), incubated at 45° C. for 1 at 45° C. for 24 hours.

^b AG=DL-alanylglycine, GA=glycyl-DL-alanine, GT=glycyl-L-tyrosine, GL=glycyl-GG=glycylglycine.

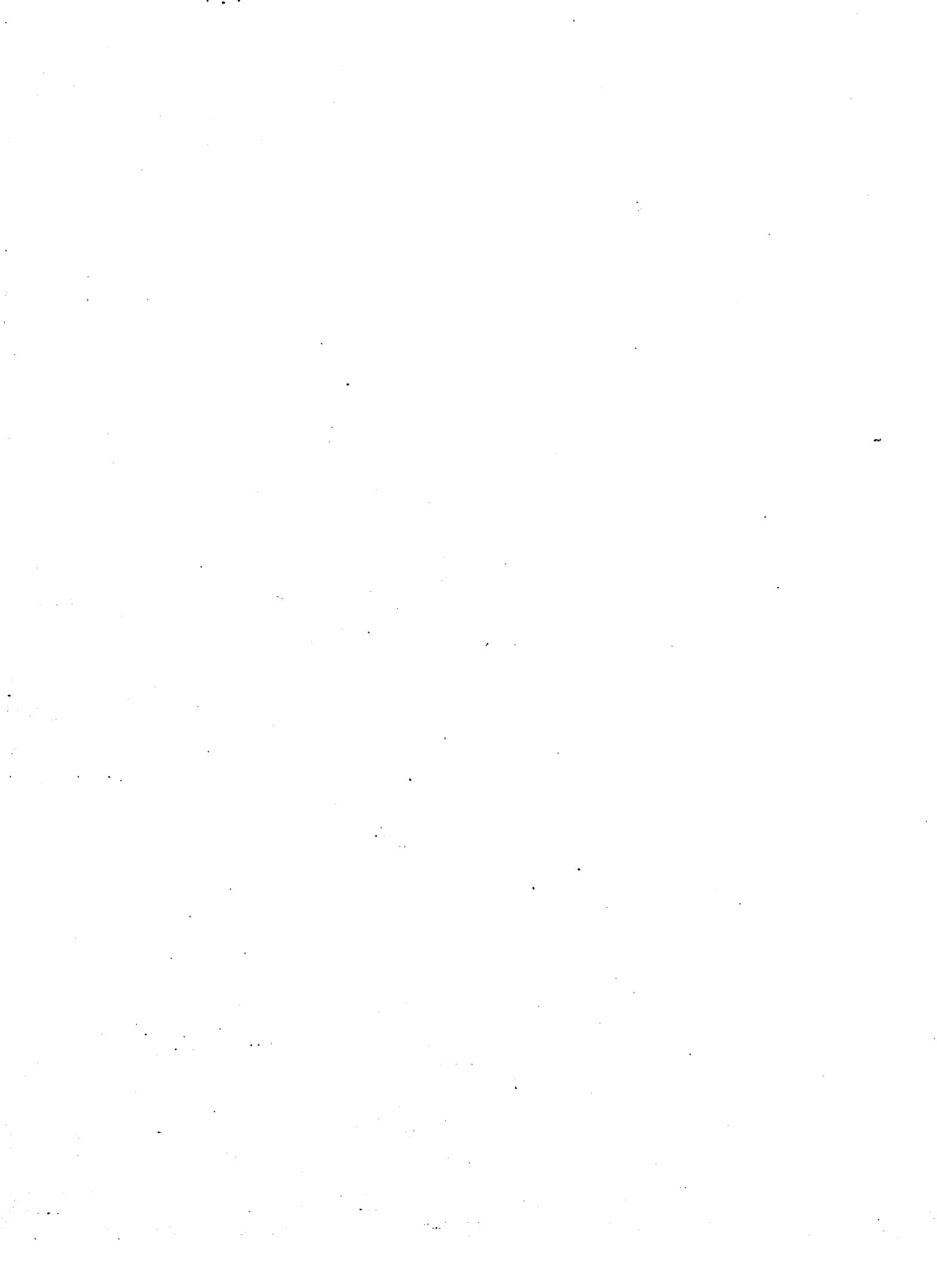
^c Cobalt concentration was 0.0004 M.



f cell-free extract and partly purified enzyme^a

of L-isomer of the following peptides ^b							Tyrosine increase from casein (γ /ml.) pH 6.0
GA pH 5.7 +CO ⁺⁺	GT pH 8.0	GL pH 8.0	LG pH 7.0	AA pH 7.0	GG pH 7.7 +CO ⁺⁺	GG pH 5.7 +CO ⁺⁺	
53	59	-	88	61	35	18	196
48	53	-	80	58	33	17	151
3	-	-	13	-	-	-	95
-	-	-	-	-	-	-	76
37	62	-	59	61	32	18	0
61	64	-	77	61	64	40	-
-	-	-	-	-	-	-	-
32	51	-	-	61	-	-	-
26	18	-	-	54	-	-	-
88	-	-	-	62	46	29	-
21	20	10	26	48	13	5	-
13	-	-	18	19	0	2	-
61	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-
18	11	14	26	32	0	5	-

t 45° C. for 1 hour. One ml. of enzyme in 5 ml. casein solution, incubated
 osine, GL=glycyl-L-leucine, LG=DL-leucylglycine, AA=DL-alanyl-DL-alanine and



DL-alanylglycine. A second washing of the precipitate using 0.1 M phosphate buffer at pH 7.0 resulted in a suspension having some proteinase activity, but no activity on DL-alanylglycine. All these results were confirmed in at least three runs.

All the steps in purification of the supernatant SIII by adsorption methods, as outlined in Figure 16, were carried out twice and some of them several times. The reproducibility of the results on peptidase activity was in most cases good. There were variations in the activities of corresponding fractions from two different runs, but fairly good agreement with respect to the ratios of activities on different peptides.

Fraction EIIb was three times as active in hydrolyzing glycyl-DL-alanine and glycyl-L-tyrosine at pH 8.0 as was fraction SEIIb, but considerably less than twice as active as fraction SEIIb in hydrolyzing DL-alanylglycine at pH 7.0 and 5.7. Similarly, the activity on glycyl-DL-alanine at pH 8.0 decreased two thirds from EIVa to EIVb whereas the activity on DL-alanylglycine at pH 7.0 decreased less than one third. Fractions EIIa, EIII and EIVc showed no activity on glycyl-DL-alanine at pH 8.0, but retained activity on DL-alanylglycine at pH 7.0. The same fractions were active on glycyl-DL-alanine and DL-alanylglycine at pH 5.7 in the presence of cobalt. The variations in activity by the different fractions on DL-alanylglycine at pH 7.0 and on DL-alanylglycine and glycyl-DL-alanine at pH 5.7 in the presence of cobalt followed mainly the same pattern, indicating that these activities may be due to the same enzyme system. Fractions EIII and

EIVc did not hydrolyze glycyglycine at pH 7.7 in the presence of cobalt. The ratios of activities on glycyglycine at pH's 7.7 and 5.7 by the different fractions usually were rather similar. Exceptions were fractions EIII and EIVc, but the activities on glycyglycine by these fractions were so low that not much emphasis could be put on these results as far as actual ratios were concerned.

Separation of proteolytic activities in cell-free extract of *L. case*
by zone electrophoresis

Several experiments with zone electrophoresis were carried out, and some separation of the proteolytic activities was obtained. The experiments were not too successful, however, since the results could not be closely reproduced. The data from one of the runs are presented in Table 11. These results probably were the most typical of the ones obtained. They were not reproducible in detail, but some of the more distinct results were confirmed in similar runs, which makes it possible to draw some conclusions from these experiments.

The experiment reported on in Table 11 was carried out with 20 ml. of cell-free extract, and was run at 150 volts at pH 6.65 for 28 hours. Seven fairly distinct zones of proteinaceous materials were observed. Zones 4 down to 1 moved toward the cathode, and zones 5, 6 and 7 moved towards the anode. The majority of the proteolytic activity was recovered from zone 7. The proteinase activity observed in zones 1 and 2 would indicate the presence of a second proteinase system in the cell-free extract. Results from other runs confirmed that at least two proteinase systems were present. The presence of peptidase activity

Table 11. Enzyme activities by extracts from the various zones after electrophoresis^a on starch

Zone	Per cent hydrolysis of the following peptides ^b at the given pH ^c									Hydrolysis of casein ^d γ/ml.
	AG pH 7.0	AG pH 5.7 + Co ⁺⁺	GA pH 8.0	GA pH 5.7 + Co ⁺⁺	GT pH 8.0	GT pH 5.7 + Co ⁺⁺	GT pH 5.7	GG pH 7.7 + Co ⁺⁺	GG pH 5.7 + Co ⁺⁺	
1	0	0	11	0	0	2	14	0	13	9
2	0	5	8	0	0	0	-	3	5	2
3	0	10	5	11	0	13	14	0	18	0
4	0	5	10	0	3	10	19	0	6	6
5	6	11	3	0	0	13	-	5	6	6
6	10	5	5	3	19	21	-	5	6	30
7	51	40	35	59	62	83	37	46	19	47

^aTwenty ml. of cell-free extract run on starch bed at 150 volts at pH 6.65 for 24 hours.

^bAG = DL-alanylglycine, GA = glycyl-DL-alanine, GT = glycyl-L-tyrosine and GG = glycyglycine.

^cTwo and one half ml. of substrate and 0.2 ml. of enzyme incubated at 45°C., DL-alanylglycine for 1 hour, the other peptides for 3 hours.

^dOne ml. of enzyme in 5 ml. of 2 per cent casein suspension at pH 6.0. Incubated at 45°C. for 48 hours. Hydrolysis expressed as increase in tyrosine (γ/ml.).

in zone 1, as observed in this run, was not too well confirmed by additional runs. The presence of a cobalt sensitive peptidase active on glycyl-L-tyrosine at pH 5.7, as indicated in Table 11, was not confirmed by the additional runs. The peptidase activities in zones 3 and 4 were found to be present in corresponding zones from other experiments, although the ratios of activities varied somewhat. The hydrolysis of glycyl-L-tyrosine at pH 5.7 by the materials obtained from zones 3 and 4 was not activated by cobalt. Two additional runs gave similar results in this respect. This indicates that there is, in addition to the one peptidase system which was strongly activated by cobalt at pH 5.7, a second peptidase system which was not activated by cobalt at this pH level.

Experiments with Cheese

Effect of cobalt on the ripening of cheese

Due to the fact that cobalt strongly activated the enzymatic hydrolysis of some peptides at a pH level similar to that found in ripening cheese, it was decided to determine if cobalt would have any effect on the quality or ripening of cheese. Two series of experimental cheeses were made. The first series was made out of 180 lbs. of milk and the second series out of 200 lbs. of milk. All cheeses in each series were treated similarly with the exception that $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ was added in various amounts in salting the cheeses. The cheeses were made according to the standard

Table 12. Effect of cobalt on the ripening of cheddar cheese. Lot I

Cheese no.	CoSO ₄ , % of fresh cheese	Score		Proteolysis	
		Flavor	Body and texture	Sol. N as % of total N	Formol N as % of total N
Two months old cheese					
Ia	0	38.5 acid 38.5 sl. acid	28.5 sl. open 28.5 weak	19.7	2.40
Ib	0.0230	38.5 sl. acid, bitter 38.0 acid, bitter	28.0 weak 28.5 weak	18.5	2.33
Ic	0.0023	38.5 acid, bitter 38.0 fermented	28.0 weak 28.5 weak	18.7	2.35
Four months old cheese					
Ia	0	37.5 fermented, bitter 38.0 fermented, bitter	28.5 sl. open, weak 29.0 weak		
Ib	0.0230	38.5 sl. fermented 39.5 sl. bitter	28.5 open, weak 28.0 sl. weak		
Ic	0.0023	38.0 sl. fermented 39.5	28.5 open, weak 28.5 sl. open, mealy		

Table 13. Effect of cobalt on the ripening of cheddar cheese. Lot II

Cheese no.	CoSO ₄ , % of fresh cheese	Score		Proteolysis	
		Flavor	Body and texture	Sol. N as % of total N	Formol N as % of total N
Two months old cheese					
IIa	0	39.0 sl. acid	28.5 sl. open		
		40.0	29.5		
IIb	0.0140	38.5 acid, sl. bitter	28.5 sl. open		
		39.5 sl. oxidized	29.0 weak		
IIc	0.0027	39.0 sl. acid	28.5 sl. open		
		39.0 sl. oxidized	29.0 weak		
II d	0.0014	39.0 sl. acid	29.0 sl. open		
		39.0 sl. oxidized	29.0 weak		
Four months old cheese					
IIa	0	39.0 sl. acid, fermented	29.0	21.7	3.03
		38.5 sl. fermented	29.0 sl. weak		
IIb	0.0140	39.5	29.0 open	21.3	3.00
		39.5	29.0 open		
IIc	0.0027	38.0 fermented	29.0 open	21.7	3.05
		38.0 fermented	28.5 open		
II d	0.0014	38.0 sl. fermented	29.0 sl. open	21.6	3.03
		38.5	29.0 sl. open		

method used at the College Creamery with the exception that 0.5 ml. of an actively growing culture of L. casei 25 was added to the cheese culture in each case. After the cheeses were taken out of the press, they were allowed to dry for 3 days, waxed and kept in a curing room at approximately 10°C. After 2 and 4 months, the cheese were scored and the cheeses in one of the series were tested for total, soluble and formol nitrogen. The amounts of cobalt added and the results from the testing of the protein breakdown in the cheeses are shown in Tables 12 and 13. According to these results, cobalt in the concentrations used had no significant effect on the ripening or quality of cheese during the first 2 months of the ripening period. With respect to the flavor, some of the cheese with added cobalt were scored as slightly oxidized, and the effect of cobalt on the flavor of the cheeses probably was not favorable. The presence of cobalt did not seem to have any effect on the body and texture of the cheese.

After 4 months the flavors of the cheeses with the highest concentration of cobalt were scored as three-fourths of a point to one point better than the controls with no cobalt added. On the other hand, some of the cheeses with lower amounts of cobalt were actually given a lower score than the cheeses with no cobalt added. Each lot of cheese was tested for the degree of proteolysis only once, and in neither case was there any significant difference in soluble nitrogen and formol nitrogen between the cheeses with and without added cobalt.

DISCUSSION

The lactobacilli have long been known to possess proteolytic enzymes (8, 10, 12, 28 and 32). The four strains of L. casei studied in this work also were found to possess high proteolytic activity, although considerable differences in proteolytic activity among the various strains were observed. These differences were most pronounced in the early stages of the incubation period, when the lactobacilli were grown in milk. The differences probably may be ascribed to the variations in growth rate, rather than to differences in enzyme production by the various strains. The addition of chalk did not have any effect on the rate of protein degradation in milk during the early stages of the incubation period, as can be seen from the results in Table 2. This may partly be due to the fact that during this period there was little difference in pH between the samples with and without chalk. Later results showed that the proteinase activity by cell-free extract of L. casei 25 was maximum at pH 5.5 to 6.0, and more than 50 per cent of the proteinase activity remained at pH 5.0. The reaction in the samples containing chalk did not drop below pH 5.0, and considerable proteinase activity was observed throughout the 9 day incubation period. In the samples where no chalk was added, and a greater drop in pH was observed, the proteinase activity in the latter part of the incubation period was much lower. In ripening cheddar cheese a reaction of approximately pH 5.0 is normal, indicating that L. casei,

if present in reasonably high numbers, could bring about considerable proteolysis in the cheese.

According to the experiments with controlled pH, maximum proteinase activity by L. casei grown in milk occurred at pH 6.5 (Figure 3). This probably is not the exact pH optimum, however, since the tests were run at intervals of one pH unit. Furthermore the average pH during the incubation period was lower than the pH at which the samples were adjusted, due to the fact that pH sometimes dropped 0.3 to 0.6 unit between adjustments. On the basis of these results and the fact that the proteinase activity at pH 7.5 was much lower than at pH 5.5, the proteinase activity by L. casei grown in milk apparently was maximum somewhere between pH 5.5 and 6.5. In a corresponding experiment with S. lactis carried out by Van der Zant and Nelson (50) the maximum activity occurred at pH 7.0 and high proteolytic activity also was obtained at pH 7.5.

The effect of temperature on the proteinase activity by L. casei seemed to depend on the length of the incubation period. With extended time of incubation the degree of proteolysis of milk in which the organism was growing increased with decreasing temperature, at least within the temperature range of 32 to 44°C., as seen in Figure 4. Several factors probably are involved in this connection, such as the effect of time and temperature on the total cell population and on the stability of the enzyme systems.

In the experiments with cell-free extracts of L. casei 25, the maximum proteinase activity occurred at progressively lower pH levels as

the temperature employed was increased (Figure 5). This tendency became more distinct as the incubation period was extended from 12 to 24 hours. Since further study (Tables 5 and 6) showed that the proteinase activity had maximum heat stability at pH 5.0 to 5.5, the explanation for the relationship of pH and temperature on the enzyme activity may be that the enzyme was more rapidly inactivated by elevated temperatures at the higher pH levels.

The activity of the proteinases at pH 6.0 was stable for at least 30 hours at temperatures up to 40°C., but maximum protein degradation during this period was obtained at 50°C. (Figures 6 and 7). At this temperature there probably is some inactivation of the enzyme, but the inactivation is more than compensated for by the increase in rate of hydrolysis. At 55°C. and pH 6.0, the enzyme was almost completely inactivated within 24 hours, as was indicated by almost no increase in protein degradation after the twenty-fourth hour.

No distinct pH optimum for the proteinase activity at 10 and 20°C. could be observed within the 24 hour period used for testing, although proteolysis was observed over a wide pH range. Baribo and Foster (4) tested the proteinase activity by cell-free extract of L. casei 25 at 11°C. at various pH levels. They used an incubation period of one week, and obtained a very distinct maximum for proteinase activity at pH 7.0, a result which is somewhat contradictory to what was found in the present study. A possible explanation is that the stability of the enzyme influences the result when such a long incubation period is used. An apparent optimum for proteinase activity may be obtained at the optimum

pH for the stability of the enzyme.

The maximum rates of increase in non-casein and non-protein nitrogen occurred at slightly different pH levels, as is seen in Figure 8. This could indicate the possible presence of two proteinase systems. The enzyme activity causing the increase in non-casein nitrogen also seemed to be more rapidly inactivated by heat at the higher pH levels than did the enzyme activity causing the increase in nitrogen soluble in 2 per cent trichloroacetic acid (Tables 5 and 6). However, these tests were carried out with two different batches of cell-free extract, which might have been the reason for the observed differences. The pH at which maximum proteolytic activity occurred in this experiment was somewhat lower than reported by Amundstad (1) and Baribo and Foster (4). It is possible, however, that the higher incubation temperature used in the present study, and the stability of the enzyme under these conditions might have influenced the results.

The pH level for maximum heat stability of the proteinases of L. casei was much lower (pH 5.0 to 5.5) than what Van der Zant and Nelson (51) observed for the proteinases of S. lactis (pH 7.0). The actual heat stability of the proteinases of L. casei was much higher than that of the proteinases of S. lactis, but at pH 5.0 it corresponded well with results obtained by Baribo and Foster (4) who worked with the same lactobacillus strain.

The high stability and activity of the proteinase of L. casei at a pH level comparable to that in ripening Cheddar cheese, indicate that this

organism might be an important factor in the protein degradation in cheese. Peterson et al. (34) found a proteinase in cheddar cheese which had maximum activity at pH 5.0. This proteinase was activated by cysteine and they indicated that it might be an endocellular bacterial proteinase. The pH level for maximum activity corresponds fairly well with that of the proteinase of L. casei, observed in the present study. The proteinase of L. casei was not found to be activated by cysteine, but the concentration used in this work was lower than the concentration Peterson et al. found activated the cheese proteinase.

Further evidence for the presence of at least two proteinase systems was indicated by the results from experiments on fractional precipitation with ammonium sulfate. The results shown in Figure 15 indicate that one proteinase fraction was precipitated at 0.55 saturation with ammonium sulfate and another at 0.7 saturation. Two fractions containing proteinase activity were actually obtained in the experiments with zone electrophoresis on starch, as can be seen from Table 11. There was considerable difference in the activities of the two fractions, the fraction which moved toward the cathode having rather low activity; however, the presence of this fraction was established by repeated runs. The possibility exists that the maximum activity of one or both of the purified fractions would be at a different pH level than observed for the crude extract, and be different from the pH which was used for testing the activities during these experiments. Tarnanen (47) was able to separate the proteolytic activities of L. casei into a proteinase and a polypeptidase,

and it is possible that one of the fractions obtained here corresponded to the polypeptidase Tarnanen found in his study.

The cell-free extract of L. casei was able to hydrolyze a series of di- and tri-peptides. The temperature optima for hydrolysis of some of these peptides varied slightly, but not enough to serve as a basis for assuming that different enzyme systems were involved. The pH optima for hydrolysis and the rates of hydrolysis varied greatly for the different peptides. The results were different from those Van der Zant and Nelson (52) obtained for hydrolysis of the same peptides by cell-free extract of S. lactis. The variations of pH optima possibly are not due only to the presence of different enzyme systems. The rates of hydrolysis of different peptides by the same enzyme may be maximum at different pH levels. One might then have a basis for assuming that the optimum pH for hydrolysis is governed by the enzyme-substrate combination rather than by the enzyme alone. The variation in pH optima, as shown in Figure 10, would otherwise indicate the presence of a whole series of peptidases. Another possible explanation is that two or more enzymes with overlapping specificities are responsible for the hydrolysis of the various substrates, and the observed pH optima are somewhere in between the real pH optima for hydrolysis by each of the enzymes present. Generally the maximum rate of hydrolysis of the glycyl-peptides occurred in the neighborhood of pH 8.0. The maximum rates of hydrolysis of the alanyl- and leucyl-peptides occurred around pH 7.0 to 7.5. The range of activity on DL-alanylglycylglycine was the most acidic studied. The rate of hydrolysis of this substrate

decreased rapidly as the reaction was raised above pH 7.0. DL-alanyl-DL-alanine, the only substrate which did not contain glycine, was rapidly hydrolyzed over a much wider pH range than the rest of the substrates. However, many more materials would need to be tested before any generalized statements relating substrate composition to differences in pH optima for peptidase activity would be justified.

L-leucylglycylglycine was rapidly hydrolyzed by the cell-free extract of L. casei 25. Davis and Smith (14) mention that this substrate has in the past been used for the assay of an "aminopeptidase", but that it is actually hydrolyzed both by an aminotripeptidase and by leucine aminopeptidase. They point out that diglycylglycine is a more specific substrate for the aminotripeptidase. Diglycylglycine was hydrolyzed very slowly by the cell-free extract of L. casei 25, so the rapid hydrolysis of L-leucylglycylglycine may according to this be ascribed to a leucine aminopeptidase. L-leucineamide is a more specific substrate for this peptidase, but was not available at the time these experiments were carried out.

According to Smith and Spackman (44), leucine aminopeptidase from swine kidney is strongly activated by manganese and less activated by magnesium. If a leucine aminopeptidase was present in the cell-free extract of L. casei 25, it either was not responsible for the hydrolysis of DL-alanylglycylglycine or any of the dipeptides tested, since the hydrolysis of these substrates was not activated by manganese or magnesium, or else this particular leucine aminopeptidase was not activated by manganese and magnesium as was the corresponding enzyme from swine kidney.

Carbobenzoylglycyl-L-phenylalanine, which is reported to be one of

the better substrates for carboxypeptidases, was not hydrolyzed by the cell-free extract of L. casei 25.

In studying the effect of metallic ions on the enzymatic hydrolysis of peptides it was found that copper, which generally is known to be inhibitory (16, 52 and 57), also in most cases was found to inhibit the peptidases of L. casei 25 (Table 7). Nickel also generally is inhibitory (52 and 57), which to some extent was confirmed in the present study, except that this ion slightly activated the hydrolysis of glycyl-DL-alanine. Manganese and magnesium ions have been reported by many workers (16, 44, 52 and 57) as being activators of different peptidases. In the present study these ions were found to have little effect on the enzymatic hydrolysis of peptides. They were in a few instances inhibitory, but did not activate the hydrolysis of any of the peptides tested.

Cobalt and to some extent zinc were found to have the greatest effect both in activating and inhibiting the hydrolysis of peptides. The effect of cobalt and zinc on the peptidase activity was therefore studied in some detail. In all but one of the cases where the effect of cobalt was tested, it activated the enzymatic hydrolysis of peptides at pH 5.5 to 6.0. The only exception was DL-alanyl-DL-alanine which was rapidly hydrolyzed at this pH level anyway. At the higher pH levels (pH 7.0 to 8.0) where the maximum rates of hydrolysis of the peptides occurred with no ions added, the hydrolysis of the alanyl- and leucyl- di- and tri-peptides was strongly inhibited by cobalt. Again the hydrolysis of DL-alanyl-DL-alanine was the exception, not being affected by cobalt. The hydrolysis of the glycyl-peptides either was activated or only slightly

inhibited by cobalt at the higher pH levels. Similar results were reported by Dudani (16) who found that cobalt strongly activated the hydrolysis of glycyl-L-leucine at pH 5.0 by cell-free extract of S. liquefaciens. The activation was less at pH 8.2. However, Dudani did not find that cobalt inhibited the hydrolysis of DL-alanylglycine at pH 7.0 or higher, as was the case in the present study.

The hydrolysis of glycylglycine was affected differently by cobalt in that it was strongly activated at pH levels ranging from pH 5.0 to 8.5. The cobalt ion is, according to Smith (42), an exceedingly specific activator for the hydrolysis of glycylglycine, and the hydrolysis of other dipeptides containing aliphatic amino acids is not influenced by cobalt. This statement does not seem to hold for the peptidases from the cell-free extract of L. casei 25, although there are some indications that the effect of cobalt depends on the presence of at least one glycine unit in the peptide. The effect of cobalt seemed to depend on whether the glycine was at the carboxy- or at the amino-terminal of the peptide. Where glycine was at the carboxy-terminal the greatest degree of inhibition by cobalt was observed at the higher pH levels. An exception was the hydrolysis of glycylglycine. Smith (41) suggests that a specific coordination compound is formed between cobalt and glycylglycine. He found that the rate of formation of this compound was maximum at pH 7.8, which also was where maximum rate of hydrolysis of glycylglycine occurred. His work does not seem to include any studies on the effect of cobalt at lower pH levels.

Smith found that the ability of glycylglycine dipeptidase to hydrolyze

other substrates depended to some extent on the ability of the substrates to form coordination compounds with cobalt. The cobalt supposedly forms a bridge between the substrate and the enzyme. Whether such coordination compounds were formed between cobalt and the substrates at the pH levels where cobalt activated the peptide hydrolysis in the present study, is not known. The coordination compound formed between glycylglycine and cobalt at pH 7.8 was characterized by a pink color (41); and it was observed during the present study that when high concentrations of cobalt were used, a pink color developed in the digestion mixtures with substrates other than glycylglycine when the reaction during the titration of the digestion mixture became alkaline.

An increase in activation by cobalt at pH 5.5 was observed as the cobalt ion concentration in the reaction mixtures increased from 0.000008 M to 0.0004 M (Figure 13). These concentrations of cobalt inhibited the hydrolysis of DL-alanylglycine and glycyl-L-tyrosine at pH's above 6.5 and 7.0, respectively. Generally the maximum rate of hydrolysis seemed to occur at progressively lower pH levels as the concentration of cobalt ions increased. These results cannot be interpreted too rigidly because tests were run only at intervals of one pH unit. Where the exact maximum rate of hydrolysis would occur is not known.

Zinc, which activated the hydrolysis of most peptides at pH 7.0 or above, seemed to eliminate the effect of cobalt on the hydrolysis of DL-alanylglycine when zinc and cobalt were added to the reaction mixture in equimolar concentrations (Table 9). A lower molar concentration of zinc decreased the effect cobalt had when added alone. There may

possibly be some competition between cobalt and zinc in the formation of a coordination compound with the substrate or the enzyme or both. Activation by zinc at pH 8.0 also was reported by Berger et al. (5) in a study of peptidases from L. mesenteroides.

Heat inactivation studies at various pH levels revealed that the peptidase activities were most stable at pH 6.0. Maximum heat stability of peptidases from cell-free extract of S. lactis was reported by Van der Zant and Nelson (52) to occur at pH 7.0; and, although the proteinases of S. lactis was considerably less heat stable than the proteinases of L. casei, the heat stability of the peptidases of these two organisms was almost the same. The results in Figure 14 indicate that the peptidase activities at pH 5.7 and in the presence of cobalt were less stable than the peptidase activities at pH 7.0 to 8.0 and in the absence of cobalt. These results may indicate the presence of at least two different enzyme systems. Other explanations for the observed difference in heat stability are possible. The heat treatment might have affected the ability of the enzyme to interact with the cobalt ions. The fact that heat stabilities in all cases were maximum at the same pH level, made it impossible to obtain any separation of the peptidase activities by means of selective heat inactivation. The peptidases had maximum heat stability at a higher pH level than the proteinases, and it might have been possible to separate these enzyme systems by means of heating at different pH levels. This was not attempted, however, since the separation was accomplished by other methods.

The experiments carried out with fractional precipitation and selective

adsorption and elution, as outlined in Figure 16, were not highly successful as far as actual separations of peptidase activities were concerned. The problem of overlapping specificities among the peptidases (15) probably made the attempted separations more difficult. It is possible that better results could have been obtained if more specific substrates had been used. The adsorption studies, however, seemed to indicate that there were at least two different peptidase systems in the cell-free extract. Glycyl-DL-alanine and DL-alanylglycine probably were hydrolyzed by different enzymes, and possibly a third enzyme system was responsible for the hydrolysis of glycylglycine at pH 7.7 in the presence of cobalt (Table 10). The characteristics of this enzyme, as far as cobalt activation and pH optimum for hydrolysis of glycylglycine were concerned, were similar to the glycylglycine dipeptidase described by Smith (41). He points out that this enzyme is extremely specific for glycylglycine, and does not hydrolyze related dipeptides.

With respect to the enzyme activity at pH 5.5 in the presence of cobalt, there is some indication that this might be one enzyme active on a variety of peptides. The pattern of relative activities on DL-alanylglycine, glycyl-DL-alanine and glycylglycine by the various fractions obtained during the course of the attempted separation, indicated that one enzyme system was responsible for the hydrolysis of these three peptides under these conditions. Whether the same enzyme system also was responsible for the hydrolysis of DL-alanylglycine at pH 7.0 in the absence of cobalt, is not known. Separation of the activity on

DL-alanylglycine at pH 7.0 from that on the same peptide at pH 5.5 in the presence of cobalt was not achieved.

The results obtained in the experiments with selective adsorption were not confirmed by the results from zone electrophoresis on the cell-free extract of L. casei 25. However, fractions were obtained which were active on glycyl-DL-alanine, but not on DL-alanylglycine. One interesting result from the work on zone electrophoresis was the isolation of one fraction which hydrolyzed glycyl-L-tyrosine at pH 5.7, but was not activated or affected by cobalt ions.

The experiments with zone electrophoresis were not too successful, since fully reproducible results were not obtained. This probably can be ascribed in part to faulty technique and insufficiently standardized methods. The preliminary runs were not as extensive as might have been desirable, and it is possible that the buffer and pH's used in these experiments were not the right ones for maximum resolution of the enzyme systems.

The generally observed fact that cobalt strongly activated the peptide hydrolysis at pH 5.5 to 6.0 led to the idea that addition of cobalt to cheese might have an effect on the ripening process. In the experiments carried out, however, little effect on the quality or protein degradation was observed during the four-month ripening period. There were slight indications of an improvement of the flavor in cheeses with the highest amount of added cobalt, and it is possible that the concentrations of cobalt ions used in these experiments were too low. The high protein content in the cheese might have tied up the cobalt ions, not making

them available for enzyme activation. It also is possible that the substrates, whose hydrolysis was activated by cobalt, either were not present in sufficient amounts to affect the ripening process, or were not important in flavor production. Further study on this point of cobalt activation of peptidases in cheese probably would be desirable.

The results obtained in this work confirm that L. casei possesses high proteolytic activity. The characteristics of the proteinases of L. casei indicate that these enzymes are active under the conditions normally found in ripening hard rennet cheeses, and that they therefore may play an important role in the protein degradation in cheese. With respect to the peptidases of L. casei, they did not have their maximum activities under the conditions normally found in ripening cheese. However, many of the peptides used for test purposes were hydrolyzed to an appreciable degree at pH levels found in ripening cheese.

SUMMARY

1. Tests for biochemical activities by four strains of Lactobacillus casei grown in skim milk, revealed that they all possessed considerable proteolytic activity, although variations in the activity by the different strains were observed.

2. Addition of chalk did not have any effect on the degree of protein degradation during the first 2 to 3 days of the incubation period. During the remainder of the incubation period a greater protein degradation was observed in the samples where chalk was added than in the samples where no chalk was added.

3. The rate of proteolysis by L. casei grown in skim milk probably was maximum somewhere between pH 5.5 and 6.5. The optimum temperature for proteolysis by L. casei grown in milk at pH 6.5 was approximately 36°C. when tests were run after 48 hours. When incubated for 96 hours, the maximum protein degradation was observed at 32°C., which was the lowest temperature used in this experiment.

4. Maximum rate of hydrolysis of casein by cell-free extracts of L. casei 25 occurred at pH levels which varied with variations in temperature. At 30°C. maximum rate of hydrolysis occurred at pH 6.5 to 7.0, and at 40 and 50°C. the maximum rate of hydrolysis occurred at pH 5.5 to 6.0. Maximum rate for increase in non-protein nitrogen occurred at slightly lower pH than did the maximum rate of increase in non-casein and amino nitrogen.

5. The optimum temperature for hydrolysis of casein by cell-free extract of L. casei 25 at pH 6.0 was approximately 50°C. The proteinase activity at 40°C. and pH 6.0 was constant for 30 hours. Almost no proteinase activity remained after an incubation period of 24 hours at 50°C. and pH 6.0.

6. Maximum heat stability of the proteinase activity occurred at pH 5.0 to 5.5. Sixty per cent of the proteinase activity remained after heating to 61.7°C. at pH 5.0 for 10 minutes.

7. The experiments with heat inactivation at different pH levels, fractional precipitation with ammonium sulfate and zone electrophoresis on starch indicated the presence of at least two proteinase systems in the cell-free extract.

8. The high activity and stability of the proteinases of L. casei at the pH level normally found in ripening hard rennet cheeses, combined with the relatively high activity at temperatures used for ripening cheese, indicate that the proteinases of L. casei may be an important factor in the protein degradation in cheese.

9. Nine di- and tri-peptides tested were hydrolyzed by the cell-free extract of L. casei 25. Maximum rate of hydrolysis occurred between pH 7.0 and 8.0. Generally the maximum rate of hydrolysis of the glycy-peptides occurred between pH 7.5 and 8.0 and of the alanyl- and leucyl-peptides between pH 7.0 and 7.5.

10. Copper, manganese and magnesium either inhibited or had no effect on the enzymatic hydrolysis of the peptides. Nickel usually inhibited or had no effect on peptidase activity, except that it activated the hydrolysis of glycy-DL-alanine. Zinc had no effect on the hydroly-

sis of the peptides at pH levels below 7.0. At higher pH levels some activation was usually observed. Cobalt had the greatest effect on the enzymatic hydrolysis of peptides. Generally it strongly activated the hydrolysis at pH 5.5 to 6.0. At pH 7.0 to 8.0 cobalt strongly inhibited the hydrolysis of alanyl- and leucyl-peptides. It activated or only slightly inhibited the hydrolysis of glycyl-peptides at this pH level. Exceptions were the hydrolysis of DL-alanyl-DL-alanine which was not affected by cobalt, and the hydrolysis of glycylglycine which was strongly activated by cobalt at pH levels ranging from pH 5.0 to 8.5.

11. The activation of cobalt at pH 5.5 decreased with decreasing cobalt concentration. Very low concentrations (0.000008 M) actually inhibited the hydrolysis of peptides at pH 5.5. The effect of cobalt on the hydrolysis of DL-alanylglycine was completely eliminated when cobalt and zinc were added to the reaction mixture in equimolar concentrations.

12. Maximum stability of the peptidase activities when heated to 61.7°C. at various pH levels occurred at pH 6.0. The peptidases were rapidly inactivated by heat at pH's 5.0 and 7.0. The peptidase activities at pH 5.5 in presence of cobalt seemed to be less heat stable than the activities at the higher pH levels in the absence of cobalt.

13. The results from the experiments with fractional precipitation and selective adsorptions indicate that DL-alanylglycine, glycyl-DL-alanine and glycylglycine possibly are hydrolyzed by three different peptidases. They also indicate that one enzyme may be responsible for the hydrolysis of several peptides at pH 5.5 in the presence of cobalt,

possibly the same enzyme as the one hydrolyzing DL-alanylglycine at pH 7.0 in the absence of cobalt.

14. Most of the peptidase activities were obtained in one fraction after electrophoresis on starch. One fraction was obtained which was active on glycyl-L-tyrosine at pH 5.5 and was not activated or affected by cobalt.

15. Maximum activities of the peptidases of L. casei occurred at a somewhat higher pH level than normally found in ripening hard rennet cheeses. However, considerable activity remained under conditions similar to those found in cheese as far as pH and temperature are concerned.

16. Some of the peptidases of L. casei were strongly activated by cobalt at pH 5.5, which is comparable to the pH in ripening cheddar cheese, but addition of cobalt in the concentrations studied to Cheddar cheese had little effect on the quality or the protein degradation in the cheese. A possible improvement in flavor after 4 months was observed in the cheeses where cobalt was added in a concentration of 0.023 per cent or higher, calculated as cobalt sulfate in per cent of fresh cheese.

REFERENCES

1. Amundstad, O. En undersøkelse over løpeenzymets og enkelte mjøkesyrebacteriers proteolytiske egenskap med saerlig henblikk p  ostens modning. (An investigation of the proteolytic activity of rennin and of some lactic acid bacteria with special reference to cheese ripening). Meddelande Nr. 28, Statens Mejerif rs k, Malm . 1950.
2. Anson, M. L. The estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. *J. Gen. Physiol.* 22:79-89. 1938.
3. Association of Official Agricultural Chemists. Official and tentative methods of analysis of the Association of Official Agricultural Chemists. 6th ed. Washington, D. C. The Association. 1945.
4. Baribo, L. E. and Foster, E. M. The intracellular proteinases of certain organisms from cheese and their relationship to proteinases in cheese. *J. Dairy Sci.* 35:149-160. 1952.
5. Berger, J., Johnson, M. J. and Peterson, W. H. The proteolytic enzymes of bacteria. I. The peptidases of *Leuconostoc mesenteroides*. *J. Biol. Chem.* 124:395-408. 1938.
6. _____, _____ and _____. The proteolytic enzymes of bacteria. II. The peptidases of some common bacteria. *J. Bact.* 36:521-545. 1938.
7. Bertho, A. and Grassmann, W. *Biochemisches Praktikum.* Berlin und Leipzig, Walter de Gruyter and Co. 1936.
8. Bertrand, G. and Weisweiller, G. Action de ferment bulgare sur le lait. *Ann. de l'inst. Pasteur* 20:977-990. 1906.
9. Block, R. J., Durrum, E. L. and Zweig, G. A manual of paper chromatography and paper electrophoresis. New York, Academic Press, Inc. 1955.
10. Braz, M. and Allen, L. A. Protein metabolism and acid production by lactic acid bacteria in milk. Influence of yeast extract and chalk. *J. Dairy Res.* 10:20-24. 1939.
11. Briggs, M. An improved medium for lactobacilli. *J. Dairy Res.* 20:36-39. 1953.

12. Christophersen, J. and Thiele, H. **Über den Einfluss von Substrat und Temperatur auf die proteolytische Aktivität einiger Kasebakterien.** Kieler milchw. Forschungsberichte 4:683-700. 1953.
13. Davis, J. G. **The use of special litmus milk media for the diagnostic cultures of lactic acid bacteria.** J. Dairy Res. 6:121. 1935.
14. Davis, N. C. and Smith, E. L. **Assay of proteolytic enzymes.** In Glick, D. *Methods of Biochemical Analysis*. Vol. 2, p. 215-257. New York, Interscience Publishers, Inc. 1955.
15. Davis, W. L., Davis, J. G., Dearden, D. V. and Mattick, A. T. R. **Studies in cheddar cheese. II. The effect of controlled oxidation reduction potential on ripening. III. The role of rennin, pepsin and lactobacilli.** J. Dairy Res. 5:144-155. 1933.
16. Dudani, A. T. **Proteolytic and coagulating enzymes of enterococci.** Unpublished Ph.D. Thesis. Ames, Iowa, Iowa State College Library. 1950.
17. Frazier, W. C., Burkey, L. A., Boyer, A. J., Sander, G. P. and Matheson, K. J. **The bacteriology of Swiss cheese. II. The bacteriology of the cheese in the press.** J. Dairy Sci. 18:373-387. 1935.
18. _____, Sanders, G. P., Boyer, A. J. and Long, H. F. **The bacteriology of Swiss cheese. I. Growth and activity of bacteria during manufacturing process in Swiss cheese kettle.** J. Bact. 27:539-549. 1934.
19. Gorbach, G. **Proteasensystem der Acidiproteolyten Gorinis.** Enzymologia 3:65-74. 1937.
20. _____ and Ulm, R. **Zur Kenntnis der Bakterienproteasen. III. Die Acidiproteolyten Gorinis.** Arch. Mikrobiol. 6:362-368. 1935.
21. Gorini, L. and Fromageot, C. **Une protéinase bactérienne (*Micrococcus lysodeikticus*) nécessitant l'ion calcium pour son fonctionnement.** Compt. rend. 229:559-561. 1949.
22. Goudot, A. **Considerations on the action of the cobalt ion in the enzymic hydrolysis of peptides.** Compt. rend. 235:1396-1398. 1952.
23. Grassman, W. and Heyde, W. **Alkalimetrische Mikrobestimmung der Aminosäuren und Peptiden.** Zeitschrift f. physiol. Chem. 183:32-38. 1929.

24. Green, A. A. and Hughes, W. L. Protein fractionation on the basis of solubility in aqueous solutions of salts and organic solvents. In Colowick, S. P. and Kaplan, N. O. *Methods in Enzymology*. Vol. 1, p. 67-90. New York, Academic Press, Inc. 1955.
25. Hull, M. E. Studies on milk proteins. II. Colorimetric determination of partial hydrolysis of the proteins in milk. *J. Dairy Sci.* 30:881-884. 1947.
26. Johnson, A. H. and Green, J. R. Modified methyl red and sodium alizarin sulfonate indicators. *Ind. Eng. Chem., Anal. Ed.* 2:2-4. 1930.
27. Korkes, S., Del Campillo, A. and Ochoa, S. Biosynthesis of dicarboxylic acids by carbondioxide fixation. IV. Isolation and properties of an adaptive "Malic" enzyme from Lactobacillus arabinosus. *J. Biol. Chem.* 187:891-905. 1950.
28. Kulp, W. L. and Rettger, L. F. Comparative study of Lactobacillus acidophilus and Lactobacillus bulgaricus. *J. Bact.* 9:357-395. 1924.
29. Kunkel, H. G. and Slater, R. J. Zone electrophoresis in a starch supporting medium. *Proc. Soc. Exper. Biol. Med.* 80:42-44. 1952.
30. Lane, C. B. and Hammer, B. W. Bacteriology of cheese. I. Effect of pasteurizing the milk on the nitrogenous decomposition of cheddar cheese. *Iowa Agr. Exp. Sta. Res. Bul.* 183. 1935.
31. _____ and _____. Bacteriology of cheese. II. Effect of Lactobacillus casei on the nitrogen decomposition and flavor development in cheddar cheese made from pasteurized milk. *Iowa Agr. Exp. Sta. Res. Bul.* 190. 1935.
32. Orla-Jensen, S. The lactic acid bacteria. København, Andr. Fred. Høst and Søn, Kgl. Hof-Boghandel. 1919.
33. _____ and Plattner, E. Beitrag zur Käseanalyse. *Landw. Jahrbuch Schweiz.* 20:421-436. 1906.
34. Peterson, M. H., Johnson, M. J. and Price, W. V. Proteinase content of cheese during making and ripening. *J. Dairy Sci.* 31:55-61. 1948.
35. Rowland, S. J. The determination of the nitrogen distribution in milk. *J. Dairy Res.* 9:42-46. 1938.

36. Schormüller, J., Wieske, R. and Winter, H. Beiträge zur Biochemie der Käsereifung. IX. Mitteilung. Die Peptidasen des Sauermilchkäses und deren Aktivität im Verlauf der Reifung. Z. Lebensm.-Untersuch. u. Forsch. 100:35-51. 1955.
37. Schwimmer, S. and Pardee, A. B. Principles and procedures in the isolation of enzymes. Adv. Enz. 14:375-409. 1953.
38. Sherwood, I. R. Lactic bacteria in relation to cheese flavor. I. J. Dairy Res. 8:224-237. 1937.
39. _____. The bacterial flore of New Zealand cheddar cheese. J. Dairy Res. 10:426-448. 1939.
40. _____. Lactic acid bacteria in relation to cheese flavor. II. Observation on the inoculation of the milk employed in cheese manufacture with lactobacilli. J. Dairy Res. 10:449-454. 1939.
41. Smith, E. L. The glycyglycine dipeptidase of skeletal muscle and human uterus. J. Biol. Chem. 173:571. 1948.
42. _____. Studies on dipeptidases. III. Hydrolysis of methylated peptides. The role of cobalt in the action of glycyglycine dipeptidase. J. Biol. Chem. 176:21-32. 1948.
43. _____. The specificity of certain peptidases. Adv. Enz. 12:191-275. 1951.
44. _____ and Spackman, D. H. Leucine and aminopeptidase. V. Activity, specificity and mechanism of action. J. Biol. Chem. 212:271-299. 1955.
45. Sode-Mogensen, M. T. Bestemmelse af ostens proteolytiske spaltningsgrad med saerligt henblik paa formoltitreringen (Determination of the degree of proteolytic decomposition in cheese with special reference to the formol titration). Meddelande Nr. 21, Statens Mejeriförsök, Malmö. 1947.
46. Sumner, J. B. and O'Kane, D. J. The chemical nature of yeast saccharase. Enzymologia 12:251-253. 1948.
47. Tarnanen, J. Proteolytic enzymes of lactic acid bacteria. Lab. Butterexportges. Valio m. b. H., Helsinki. 1930. (Original not seen; abstracted in Chem. Abstr. 24:4529. 1930.)
48. Tiselius, A. Zone electrophoresis in filter paper and other media. Discussions Faraday Soc. 13:29-33. 1953.

49. Tittsler, R. P., Sanders, G. P., Lochry, H. R. and Sagers, O. S. The influence of various lactobacilli and certain streptococci on the chemical changes, flavor development and quality of cheddar cheese. (Abstract). *J. Dairy Sci.* 31:716. 1948.
50. Van der Zant, W. C. and Nelson, F. E. Proteolysis by *Streptococcus lactis* grown in milk with and without controlled pH. *J. Dairy Sci.* 36:1104-1111. 1953.
51. _____ and _____. Characteristics of an endocellular proteolytic enzyme system of *Streptococcus lactis*. *J. Dairy Sci.* 36:1212-1222. 1953.
52. _____ and _____. Characteristics of some endocellular peptidases of *Streptococcus lactis*. *J. Dairy Sci.* 37:795-804. 1954.
53. _____ and _____. Amino acids and peptides in the protein-free fraction of milk before and after incubation with *Streptococcus lactis*. *J. Dairy Sci.* 37:790-794. 1954.
54. Virtanen, A. I. and Lundmark, E. Über das Wesen der Caseinspaltung durch Milchsäurebakterien. *Milchw. Forsch.* 8: 375-382. 1929.
55. Weichselbaum, T. E. An accurate and rapid method for the determination of proteins in small amounts of blood serum and other plasma. *Am. J. Clin. Path.* 16:40-49. 1946.
56. Wetter, L. R. Proteolytic enzymes of microorganisms. VI. The separation of proteases from *Mortierella renispora* by zone electrophoresis. *Can. J. Med. Sci.* 32:20-26. 1954.
57. Zimmerman, H. K. Proteolytic enzymes of ripening cheddar cheese. Unpublished Ph.D. Thesis. Ames, Iowa, Iowa State College Library. 1950.
58. Zittle, C. A. Adsorption studies of enzymes and other proteins. *Adv. Enz.* 14:319-374. 1953.

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