PRODUCTION OF A PROTEIN HYDROLYSATE BY FERMENTATION OF MILK USING *BACILLUS CEREUS* WITH A STUDY OF THE PROTEOLYTIC ENZYME INVOLVED

by

DENNIS HUGH COLE

B. S., University of Missouri, 1966

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

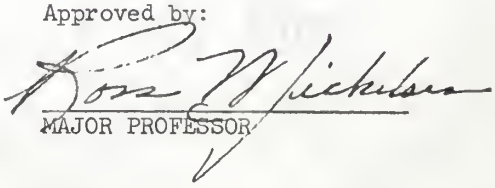
Department of Dairy and Poultry Science

KANSAS STATE UNIVERSITY

Manhattan, Kansas

1968

Approved by:



MAJOR PROFESSOR

TABLE OF CONTENTS

INTRODUCTION

The hydrolysis of proteins, or proteolysis, consists of splitting protein molecules into their constituent parts. There are three basic methods used; namely acid, alkaline, and enzymatic hydrolysis. Acid hydrolysis is the most commonly used method. Alkaline hydrolysis causes the complete destruction of several amino acids which limits the usefulness of this method. Enzymatic hydrolysis has been used extensively in studies of protein structure due to the specificity of some enzymes for certain peptide linkages. However, this method has found only limited use in the production of protein hydrolysates for food uses.

Microbial fermentation is a well established method of preparing numerous food products such as bread, alcoholic beverages, vinegar, fermented vegetables, and fermented milk products. Fermentations primarily are enzymatic processes which occur as a result of microbial metabolism. The hydrolysis of proteins by microorganisms is the result of action of extracellular and endocellular proteolytic enzymes produced by the organisms. The activity of proteolytic enzymes on milk proteins traditionally has been considered undesirable in the dairy industry, except in the manufacturing of various cheeses. However, by using controlled methods of fermentation, followed by certain processing procedures, it is possible to produce a desirable protein hydrolysate from milk (7).

The manufacture of protein hydrolysates for use in food products is not an entirely recent innovation. The well known soya sauce, the original protein hydrolysate, was produced by natural fermentation many years ago by people of the Orient. It has only been within comparatively recent years, however, that protein hydrolysates have been produced commercially for food uses. Hydrolyzed proteins now are utilized widely in the food industry as flavor enhancing

ingredients. Hydrolysates are used mainly to accentuate or simulate meat flavors in foods, such as certain processed meat products, snack foods, and soups, or they may be used to provide background flavors in other food items, such as canned vegetables, sauces, and dips.

The objectives of the study reported in this thesis were to: (a) select a highly proteolytic microorganism and study its proteolytic activity in milk, (b) evaluate the characteristics of the protein hydrolysate, and (c) produce, increase the purity, and study the properties of the proteolytic enzyme.

REVIEW OF LITERATURE

Production, Use, and Characteristics of Protein Hydrolysates

Protein hydrolysates usually are prepared by acid hydrolysis of proteins from either plant or animal sources. Some of the first work of this kind was described over twenty years ago by Hall (14). He discussed the acid digestion of a mixture of casein, corn, and wheat gluten which yielded a protein hydrolysate with "a meaty or other flavorful taste". The protein digest was clarified, neutralized, and dried for addition to soups, sauces, meat products, and spice mixtures.

Connell (8) recently reviewed the methods of manufacturing, chemical composition, and uses of hydrolyzed proteins in the food processing industry. Although acid hydrolysis currently is the most common method used by processors of protein hydrolysates, Connell thought that successful procedures would be developed for manufacturing enzymatic protein hydrolysates. It was noted that the annual consumption of hydrolyzed proteins in the United States is about 30 million pounds. These hydrolysates are utilized in meats, soups, baked goods, and numerous other food products.

Zeimba (34) discussed the production of hydrolyzed vegetable proteins (HVP) by acid hydrolysis in a modern facility in which over 1,000 lb. of hydrolysates per hour could be produced. Processing procedures consisted of acid hydrolysis of the proteins, followed by neutralization, filtration, decolorization, concentration to 45 or 85% solids, and spray-drying. Fourteen types of hydrolysates were processed which permitted blending to produce a total of 50 different kinds of HVPs. Three reasons were listed as to why increasing amounts of protein hydrolysates are being used in foods: (a) HVPs have a "meaty" flavor which makes it easier to replace costly, difficult-to-process beef extract, (b) HVPs can compensate for natural flavors lost during certain processing steps, and (c) flavors of HVPs are not lost or changed during prolonged storage.

Malkames and Walter (20) used a proteolytic enzyme to hydrolyze the proteins precipitated from cheese whey. The hydrolyzed product was blended with sour cream, cream cheese, or cottage cheese to make various types of food spreads.

Murray and Baker (24) investigated the influence of various factors such as type of protein, extent of hydrolysis, and nature of enzyme preparation on the flavor of enzymatic protein hydrolysates. It was found that the flavor of the hydrolysates varied from very bland to extremely bitter with some having a meat-like flavor. The type of proteolytic enzyme used in the preparation of casein hydrolysates was shown to affect the intensity of bitterness in the resulting products. The flavor of a casein hydrolysate was greatly improved by treating it with activated carbon for two hours followed by filtering to remove the carbon. Further studies at the same laboratory by Carr et al. (6) were conducted to determine the cause of the bitterness in casein hydrolysates. Treating casein with dilute sodium hydroxide before digestion with a proteolytic enzyme removed orthophosphate which reduced the bitterness in the hydrolysates

in direct proportion to the amount of orthophosphate removed. A series of fractionations of a tryptic casein hydrolysate yielded an extremely bitter fraction. Chromatographic and electrophoretic studies indicated that a polypeptide was the cause of the bitterness.

Claydon (7) reported on some general characteristics of a hydrolysate of nonfat milk solids produced by Pseudomonas fluorescens. By following certain processing procedures, the preparation gave a broth-like product that resembled beef extract. Further work was conducted by Mickelsen et al. (23) on some chemical and flavor characteristics of a milk proteolysate produced by Ps. fluorescens. The proteolysate from different processing stages was fractionated by gel filtration with subsequent analysis of the fractions to determine the possible relationship between flavor changes and chemical characteristics. The typical beef broth-like flavor was isolated and was found to contain seventeen amino acids, carbohydrates, phosphates, and products of the browning reaction.

Bacterial Proteolytic Enzymes

The number of bacterial species which produce proteolytic enzymes is quite large. Some of these bacteria are considered to be highly proteolytic, whereas others are only moderately or slightly proteolytic. In this review, major emphasis will be placed on those organisms that are considered highly proteolytic.

The genus Bacillus would appear to be one of the most prominent among all bacterial genera in regard to proportion of species that produce proteolytic enzymes. McConn et al. (21) purified a Bacillus subtilis proteinase from a commercial enzyme preparation. The enzyme was referred to as a neutral proteinase, since the pH optimum was found to be near pH 7.0. The temperature for maximum activity was 58 C in the presence of calcium acetate, but about 50 C in the absence of calcium salts. Chelating agents and heavy metal ions inhibited

the enzyme, but sulfhydryl blocking reagents and diisopropylphosphorofluoridate had no effect on its activity. A direct relation existed between the specific activity of the enzyme and its zinc content. Microchemical analyses indicated that the enzyme contained 1 g atom of zinc per mole.

Babbar et al. (2) reported the preparation and properties of an acidic endopeptidase produced by a variant of B. subtilis. The enzyme was purified about 25-fold by fractionation with ammonium sulfate. The pH optimum of this enzyme was found to be pH 5.5, in contrast to the pH optimum of endopeptidases from other organisms reported to be neutral or alkaline to pH 10.0. Furthermore, the acidic optimum pH was significant because the enzyme had been used in the manufacture of Cheddar cheese (31). Chelating agents did not inhibit the activity of the enzyme, but sulfhydryl inhibitors and trypsin inhibitors completely inhibited its action.

Grossman (13) made a study of the proteolytic enzymes produced by Bacillus pumilus, Bacillus subtilis, Bacillus subtilis var. aterrimus, and Bacillus cereus. The enzymes were isolated by precipitation with ethanol and ammonium sulfate. The proteolytic activity of B. pumilus was found to be greater than that of the other species studied. The optimum pH for activity of all the enzymes was pH 7.5, but the optimum temperature ranged from 37 to 50 C.

Emanuiloff (12) found that a proteolytic enzyme from Bacillus mesentericus hydrolyzed a large number of proteins of vegetable and animal origin. The enzyme possessed a milk clotting ability which was at a maximum at 65 C and pH 6.5. Salts of heavy metals inhibited while $CaCl_2$ and cysteine activated the enzyme's milk clotting activity.

O'Brien and Campbell (27) isolated and studied a proteolytic enzyme produced at 55 C by Bacillus stearothermophilus. A 20- to 40-fold increase in specific

activity was achieved by means of fractionation with ammonium sulfate, calcium phosphate gel treatment, and precipitation with ethanol. The enzyme was most active at pH 6.9 to 7.2 and calcium or manganese was required for activity on casein. Sulfhydryl inhibitors at 10^{-4} M inhibited 90% of the enzyme activity.

Damordaran et al. (10) observed that Bacillus licheniformis produced a powerful protease in a medium containing peanut meal. The enzyme was precipitated with acetone or ethanol and was found to have a greater activity than commercial trypsin. Both proteinase and peptidase activities were present and the properties of each were investigated. The proteolytic activity was neither activated nor inhibited by cysteine, cyanide, or iodoacetate but bivalent metals caused a slight inhibition.

Bernlohr (3) studied the characteristics of an extracellular protease produced by presporulating cells of B. licheniformis. The enzyme was found to have a broad range of pH optima and it was most active at 67 C. Metal cofactors were not required by the enzyme for activity and it did not contain an essential sulfhydryl group. Results of immunological procedures suggested that the protease was synthesized by the cell only after growth had been completed.

Hall et al. (14) found that culture filtrates of B. licheniformis contained three types of enzymes. One of the enzymes exhibited mainly proteolytic activity; another was mainly esterolytic; and the third was an aminopeptidase. The enzymes were differentiated by the use of synthetic substrates, organofluoride inhibitors, and heat denaturation studies. Proteolysis by the unfractionated enzyme system was inhibited by disodium ethylenediaminetetraacetate (EDTA), but was enhanced by calcium.

Melachouris and Tuckey (22) reported recently on the properties of a milk-clotting enzyme isolated from a culture of B. cereus. The action of the enzyme

on casein and skimmilk was investigated and compared to that of calf rennet. It was found that the clotting ability of the microbial enzyme could be increased by adding calcium ions, increasing temperature, and decreasing pH. However, the clotting ability of calf rennet was greater under the same conditions. Degradation of casein, as measured by determining nonprotein nitrogen, was more rapid by the microbial enzyme than by calf rennet.

Salter (29) purified and studied some properties of a proteinase from B. cereus, NCTC 945. The enzyme was purified by precipitation with ammonium sulfate and by diethylaminoethyl (DEAE) cellulose. The enzyme showed greatest activity at pH 6.8 in phosphate or tris buffers containing 10^{-4} M Ca^{++}. The enzyme was completely inhibited by 10^{-3} M Co^{++}, Zn^{++}, or Fe^{++}, and partially inhibited by 10^{-3} M Mn^{++} or Mg^{++}, and $4x10^{-3}$ M cysteine.

Neumark and Citri (25) observed marked variations in the amount of protease produced by B. cereus which was attributed to differences in the composition of the growth medium. They found that this organism produced 20 times as much proteolytic activity in a medium containing 1% casein hydrolysate as in a medium containing 2% casein hydrolysate. Furthermore, it was found that the yield of protease was strongly suppressed by adding 1 mg/ml of each of the amino acids glycine, alanine, leucine, isoleucine, valine, aspartic acid and glutamic acid to the medium containing 1% casein hydrolysate. Thus the results indicated that high levels of certain amino acids inhibited protease production by this organism. More recently, Levisohn and Aronson (18) made additional studies on this phenomenon of B. cereus. They found that both protease production and sporulation could be inhibited by high concentrations of a mixture of amino acids. The results suggested that protease production was regulated by the level of one or more catabolic or biosynthetic intermediates.

Many organisms of the genus Pseudomonas have been found to produce proteolytic enzymes. Peterson and Gunderson (28) found that a culture of Pseudomonas fluorescens had a very active enzyme system consisting of endocellular and extracellular proteolytic enzymes. The greatest amount of extracellular enzyme production occurred at 0 C and the amount produced decreased with increasing temperature to 30 C. However, once the enzyme was liberated, proteolytic activity was accelerated by increased temperature. Greater amounts of enzyme were produced at pH 7.0 and 8.5 than at other values between pH 5.5 and 9.0. The elaborated enzyme had peaks of optimum activity at pH 6.2, 7.8, and 8.8.

Hurley et al. (17) found that Ps. fluorescens produced a proteolytic enzyme system that was active against hemoglobin and spray-dried egg white. The enzyme preparations reached maximum activity at 37 C in egg white and at 25 C in hemoglobin at pH values between 7 and 9. Purification of the enzyme was achieved by fractionation with ammonium sulfate and DEAE-cellulose chromatography. The proteolytic activity of the enzyme preparations was greatly increased when the enzyme was preincubated with 0.01 M ferrous chloride.

Van Der Zant (32) and Camp and Van Der Zant (5) studied the extracellular and endocellular proteolytic enzymes of Pseudomonas putrefaciens. Maximum activity of the extracellular enzymes on casein and lactalbumin occurred at pH 7.0 to 8.0. The optimum temperature for activity on casein was 37 C, but lactalbumin was more readily hydrolyzed at 45 C. Reducing agents and metallic ions had no effect on the proteolytic activity of the extracellular enzymes. The endocellular proteolytic enzyme system showed the presence of several peptidases. Certain metallic ions stimulated the activity of some peptidases whereas other ions were inhibitory.

EXPERIMENTAL PROCEDURES

Selection of a Proteolytic Organism

Source of organisms. Cultures of previously isolated, unidentified proteolytic bacteria were obtained from the Food Products Laboratory collection. Other proteolytic organisms were isolated from samples of Cottage cheese, butter, and soil. Isolation of the organisms was attained by streaking the samples on plates of Standard Plate Count agar containing 7% sterile litmus milk. The inoculated litmus milk tubes were incubated at 25 and 37 C until proteolysis was evident and then were stored at 5 C.

Culture propagation and media. Stock cultures of proteolytic organisms were propagated in litmus milk with periodic transfers to maintain culture viability.

Litmus milk used for milk agar plates and for culture propagation was prepared by reconstituting nonfat dry milk (NFDM) to 10% solids, adding litmus, and autoclaving. Milk for determination of microbial proteolytic activity and for production of protein hydrolysates was low-heat NFDM reconstituted to 10% solids. The reconstituted NFDM was dispensed in 250-ml quantities into 1-liter Erlenmeyer flasks. All media were autoclaved for 15 minutes at 15 psi.

Determination of amount of proteolysis. Flasks of sterile reconstituted NFDM were inoculated with 1 ml of the proteolytic stock cultures. The milk was incubated at either 25, 32, or 37 C for the desired period of time. At regular intervals, usually every two days, a sample was removed from the proteolyzed milk and the extent of proteolysis was measured.

Two methods were used for measuring the amount of proteolysis: (a) ultraviolet absorption of trichloracetic acid (TCA) filtrates at 280 mμ, and (b) quantitative ninhydrin.

TCA filtrates were prepared by adding 9 ml of 10% TCA to 1 ml of proteolysate. Unhydrolyzed proteins were precipitated and filtered out by filtration through Whatman No. 3 filter paper. Four ml of 10% TCA were added to 1 ml of the filtrate prior to determining the absorbancy at 280 mμ using a Beckman Model DU spectrophotometer.

The procedures used for the quantitative ninhydrin method were the same as used by Mickelsen et al. (23). A 1 ml sample of proteolysate was diluted 1:250 in distilled water. One ml of the diluted proteolysate was used in the ninhydrin test. Absorbancy at 570 mμ was determined on a Bausch and Lomb Spectronic 20.

By following the above procedures, relative amounts of proteolytic activity produced by the organisms were determined. Results of the analyses were compared and a highly proteolytic culture was selected for further study.

Identification of organism. The proteolytic organism selected was observed to be an aerobic sporeforming rod. Further characteristics of the organism were determined by the procedures of Smith et al. (30) and the results were found to be nearly identical to those listed for Bacillus cereus by these workers and by Breed et al. (4).

Evaluation of Protein Hydrolysate

Production of protein hydrolysate. The procedures used for production and evaluation of the protein hydrolysate were variations of those described by Mickelsen et al. (23). Sterile reconstituted NFDM was inoculated with 1 ml of B. cereus litmus milk culture and incubated at 37 C for 7 days. After the incubation period, the proteolysate was inoculated with a mixed culture of Lactobacillus bulgaricus and Streptococcus thermophilus and incubation was continued at 37 C for fermentation of lactose. Lactic acid produced was

neutralized to pH 6.0-6.5 by daily addition of 3 N NaOH. When fermentation of lactose had ceased, usually after 4 days as indicated by no further decrease in pH, the proteolysate was further processed. Processing of the proteolysate consisted of: (1) adjusting pH to 5.0, (2) adding activated carbon, followed by heating, shaking, and finally centrifuging to remove the carbon, (3) vacuum treating supernatant, (4) autoclaving for 10 minutes at 15 psi, and (5) concentrating to one-fourth the original volume. The product was evaluated for flavor and odor by laboratory personnel. In some cases, proteolysate produced by other organisms was processed in an identical manner for comparative purposes.

Effect of temperature. The effect of temperature on the rate of protein hydrolysis and flavor of the proteolysate was determined by incubating flasks of reconstituted NFDM inoculated with B. cereus at 25, 32, and 37 C for 9 days. The amount of proteolysis produced at each temperature was determined at regular intervals during the incubation period. Following the incubation period, the three flasks of proteolysate were processed and evaluated organoleptically for flavor and odor.

Preliminary chemical and flavor characteristics. Sephadex G-25 was used to fractionate the proteolysate at each of three main processing stages, i.e., 7-day proteolysate, 11-day proteolysate, and processed concentrate. Eight ml of each product was passed through a 2.50- by 36-cm column of Sephadex G-25, using 0.02 M NaCl as eluant. Five-ml fractions were collected and the absorbancy was determined at 280 mμ with a Beckman Model DU spectrophotometer. The 5-ml fractions were autoclaved and evaluated for flavor characteristics.

Enzymatic Studies

Enzyme Production. The medium initially used for proteolytic enzyme production by B. cereus was sterile reconstituted NFDM. It became evident

that this would not be a suitable medium for enzyme production for two reasons: (a) the amount of enzyme produced was relatively small, and (b) purification of the enzyme from a medium containing a complex of proteins proved to be quite difficult. Therefore, a different medium for enzyme production was selected.

In preliminary work it was observed that B. cereus produced substantially more proteolytic enzyme in Cheddar cheese whey than in milk. Since Cheddar cheese whey is less complex than milk, it was selected as the medium for production of the proteolytic enzyme from B. cereus.

Fresh whey was obtained from a local cheese factory and was dispensed in 2-liter quantities into 4-liter Erlenmeyer flasks. The whey was adjusted to pH 6.5 by addition of 3 N NaOH and was sterilized in an autoclave at 15 psi for 15 minutes.

Each flask of whey was inoculated with 5 ml of a 24-hour litmus milk culture of B. cereus and incubated at 37 C for seven days. The amount of enzyme produced and the extent of proteolysis in the whey were determined at regular intervals during the seven-day period of incubation. The results were compared to those obtained from the same analyses made on reconstituted NFDM under identical conditions. The amount of enzyme produced was determined by procedures described in the next section. The quantitative ninhydrin method, discussed in the section on determination of amount of proteolysis, was used to measure the extent of proteolysis. At the end of the incubation period, the flasks were stored at 5 C until the hydrolysate was fractionated.

Enzyme assay. Proteolytic activity was determined by a modification of the procedures of Anson (1). One ml of enzyme preparation was added to 5 ml of a 1% solution of casein. The samples were incubated at 37 C for exactly 30 minutes before 5 ml of 26.4% TCA were added to stop the reaction and to

precipitate the unhydrolyzed proteins. After the tubes were allowed to remain in the water bath for approximately 30 minutes, the contents of the tubes were filtered through Whatman No. 3 filter paper. The extent of proteolysis was determined by reading the absorbancy of the filtrates at 280 mμ in a Beckman Model DU spectrophotometer. Blanks were prepared by adding 5 ml of TCA to 5 ml of substrate, followed by addition of 1 ml of enzyme preparation. One unit of enzyme activity was arbitrarily defined as the amount of enzyme that produces a 0.1 increase in absorbancy at 280 mμ in 30 minutes at 37 C.

Casein substrate was prepared by adding 10 g of vitamin-free casein to approximately 100 ml of 0.01 M sodium phosphate buffer, pH 7.0. Two ml of 3 N NaOH were added and the mixture was heated for 15 minutes in a boiling water bath until the casein was solubilized. After cooling, the volume was adjusted to 250 ml with phosphate buffer making a final concentration of 4% casein. Two hundred mg of sodium azide were added as a preservative. The above preparation was diluted 1:4 with phosphate buffer before use.

For determination of specific activities, the protein concentration of the enzyme preparations was determined by the method of Lowry et al. (19), using casein as a standard. Absorbancy of the samples was read at 650 mμ on a Bausch and Lomb Spectronic 20. Protein concentrations were read from a standard curve.

Enzyme Fractionation. All steps of the fractionation procedures were performed at 5 C.

Whey media used for enzyme production was centrifuged at 11,000 x g for 20 minutes in a refrigerated centrifuge to remove cellular material and insoluble protein. A 2:1 volume of cold 95% ethanol was added to the supernatant to precipitate the proteins. The mixture was allowed to stand for about 12 hours and then was centrifuged at 7500 x g for 20 minutes. The precipitate was

collected, resuspended in distilled water, and dialyzed against distilled water for 24 hours. After dialysis, the volume was adjusted to approximately 15% of the original volume with distilled water.

The dialyzed ethanol precipitate was brought to 25% saturation with ammonium sulfate. The mixture was allowed to stand for several hours before it was centrifuged and the precipitate was discarded. The supernatant was filtered through Whatman No. 41 filter paper and the filtrate was increased to 75% saturation by addition of ammonium sulfate. After standing overnight, a precipitate formed which was collected by centrifugation. The precipitate was resuspended in 0.01 M sodium phosphate buffer, pH 7.0 and dialyzed against this buffer to remove the ammonium sulfate. Insoluble material present after dialysis was removed by centrifugation and filtration. The dialyzed enzyme preparation was frozen and stored until needed. Samples were removed for analysis of protein content and enzyme activity during the various steps of fractionation.

Attempts to purify the enzyme further by gel filtration using Biogel A 5-M, Sephadex G-100 and G-200, and by ion exchange chromatography using DEAE-cellulose were unsuccessful.

Enzyme characteristics. The effect of the following were studied on the proteolytic characteristics of the enzyme: (a) temperature, (b) pH, (c) substrate concentration, and (d) activators and inhibitors.

The effect of temperature on proteolytic activity was determined by incubating the enzyme with casein substrate at temperatures ranging from 25 to 65 C. The amount of activity produced at eight different temperatures was measured by the procedures described for enzyme assay.

The enzyme was assayed for optimum proteolytic activity at eight pH values ranging from pH 5.0 to 9.0. McIlvaine buffers of constant ionic strength were

prepared at pH 5.0, 6.0, 6.6, and 7.2 according to the procedures of Elving et al. (11). Tris (hydroxymethyl) aminomethane-HCl (tris-HCl) buffers were prepared at pH values of 7.5, 8.0, 8.5, and 9.0. The ionic strength of all buffers was 0.5. A 1% hemoglobin substrate was prepared in each of the buffers. The procedures for determining the amount of proteolytic activity at the various pH values were the same as described under the enzyme assay section, except that hemoglobin was used as substrate and the samples were incubated for one hour at 37 C rather than for 30 minutes.

Three different proteins were prepared at six levels of concentration to determine the effect of increasing substrate concentration on amount of proteolytic activity produced by constant amounts of enzyme. Twenty mg/ml (2%) of casein, β-lactogobulin, and α-lactalbumin were prepared in 0.01 M tris-HCl buffer, pH 7.5. Appropriate dilutions of each protein were made to make the following levels of substrate (mg/ml): 0.5, 1.0, 3.0, 5.0, and 10.0. One ml of each substrate at these concentrations was incubated with 1 ml of enzyme preparation and the amount of proteolytic activity was measured by the standard assay procedure.

The activity of the proteolytic enzyme was determined in the presence of certain metal ions and other compounds to test for activation or inhibition. One ml of an enzyme preparation, which had been extensively dialyzed in double distilled water, was preincubated for 1 hour at 37 C with 1 ml of 0.01 M solutions of the following compounds prepared in double-distilled water: $CaCl_2$, $CuCl_2$, $FeSO_4$, $MgSO_4$, $MnSO_4$, $ZnSO_4$, EDTA, potassium oxalate, cysteine, and iodoacetamide. Diisopropylfluorophosphate (DFP) was used in the same manner at a concentration of 0.005 M. Four ml of substrate were added after the 1-hour incubation period. Incubation was continued for 30 minutes and the amount of enzyme activity was measured by standard assay procedures.

RESULTS AND DISCUSSION

Selection of a Proteolytic Organism

Eleven pure cultures of proteolytic bacteria were obtained from the laboratory collection or by isolation procedures. These organisms were inoculated into litmus milk and incubated at various temperatures for observation of growth and proteolytic activity. The amount of proteolysis produced by the different organisms varied from slight to almost complete proteolysis of the litmus milk. From these eleven cultures, four cultures were selected, based on amount of proteolysis, for further study of their proteolytic activity in milk. Since Ps. fluorescens was used for production of proteolysate in work by Claydon (7) and by Mickelsen et al. (23), the amount of proteolytic activity produced by this organism was also determined for comparative purposes.

The different species were inoculated into flasks of sterile reconstituted NFDM and incubated at their optimum growth temperatures and the extent of proteolysis was determined. Figure 1 shows the relative amount of proteolysis produced by the four different species and by Ps. fluorescens as determined by the quantitative ninhydrin method. Two of the organisms, both of which were aerobic sporeformers, produced enzymes capable of extensive proteolytic activity compared to those of Ps. fluorescens and a Gram-negative rod, both of which showed moderate proteolytic activity. The other species studied, which produced acid and proteolysis, was much less proteolytic than any of the other organisms.

The overall objective of this research was to find a highly proteolytic organism which would lend itself to the production of a protein hydrolysate from milk. To satisfy this requirement, the proteolysate resulting from the fermentation had to have desirable flavor and odor characteristics after final processing. With this in mind, the proteolysates produced by the two aerobic

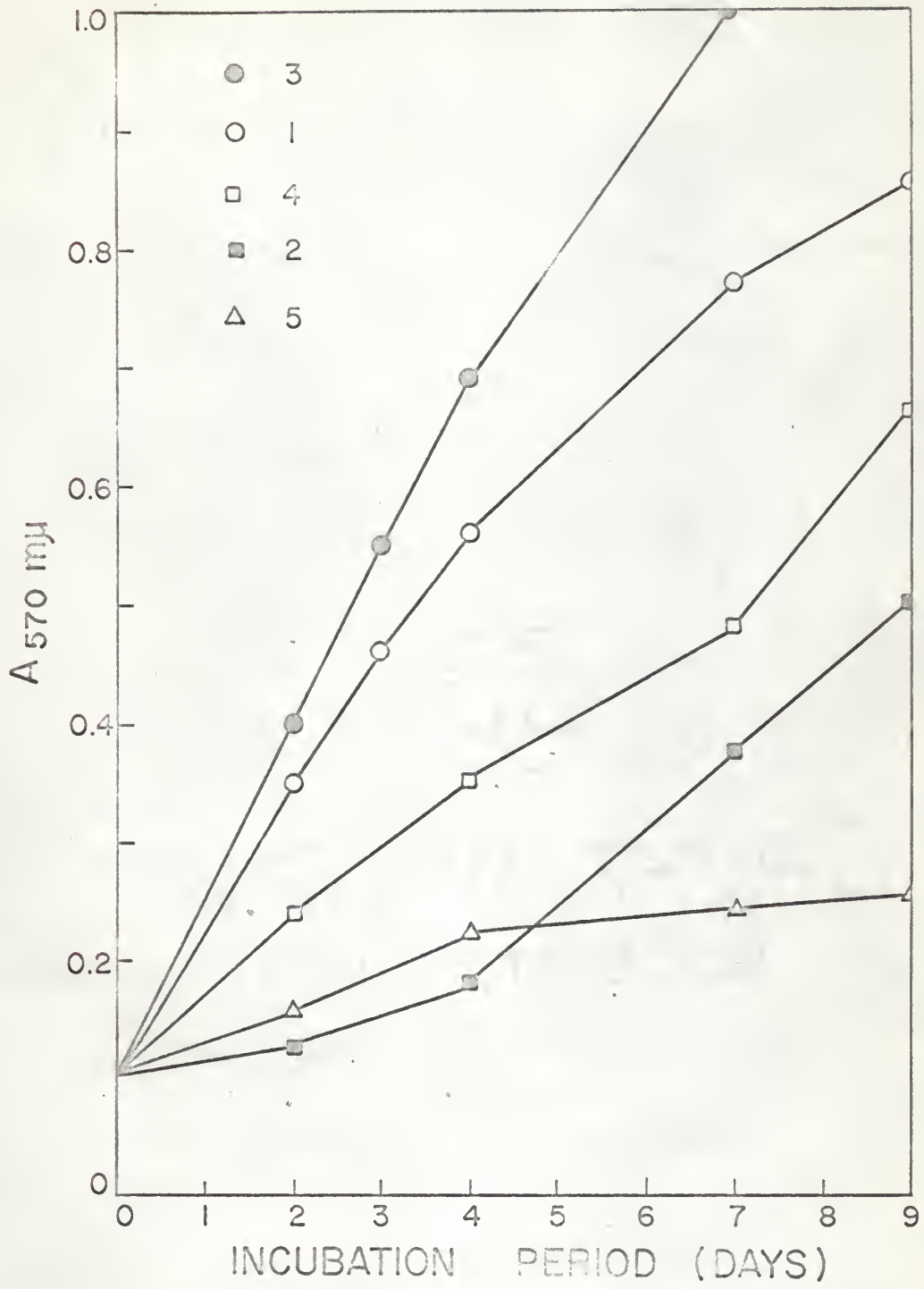


Figure 1. Relative amount of proteolysis produced by five organisms in 10% solids reconstituted NFDM. No. 1, aerobic sporeformer from culture collection; No. 2, Ps. fluorescens; No. 3, aerobic sporeformer isolated from butter; No. 4, Gram-negative rod; No. 5, acid-proteolytic Gram-positive cocci.

sporeformers and Ps. fluorescens were processed and evaluated for flavor and odor. The processed proteolysate of the aerobic sporeformer isolated from butter was found to have a very undesirable flavor, thus this organism was not acceptable for further work. The other aerobic sporeformer produced a desirable processed proteolysate with flavor characteristics similar to those of Ps. fluorescens. Furthermore, as is illustrated in Figure 1, the aerobic sporeformer hydrolyzed milk proteins at a much faster rate than did Ps. fluorescens. The aerobic sporeformer was selected for further study.

Further characteristics of the aerobic sporeformer were determined and are shown in Table 1. Based on the identifying characteristics, the organism was identified as Bacillus cereus.

Table 1. Characteristics of the aerobic sporeformer.

1.	Gram-positive, sporeforming rod.
2.	Sporangia not swollen.
3.	Young cells grown on glucose agar were vacuolated.
4.	Growth on agar--white, spreading, arborescent colonies.
5.	Gelatin hydrolysis--positive.
6.	Starch hydrolysis--positive.
7.	Acetylmethylcarbinol production--positive.
8.	Motility--positive.
9.	Nitrites produced from nitrates--negative.
10.	Acid from glucose, sucrose, and glycerol--positive.
11.	Acid from xylose, arabinose, lactose, and mannitol--negative.

Some questions have been raised relative to the possible pathogenicity of B. cereus (16). Dack et al. (9) examined the food poisoning potentialities of four strains of B. cereus by feeding these organisms and/or their products of growth to human volunteers. Under the experimental conditions employed in the study, this organism did not seem capable of causing food poisoning.

In a later study, Nygren (26) stated that when B. cereus had the opportunity to multiply to a suitable extent in foods containing lecithin, a type of food poisoning may result. B. cereus produces phospholipase C which hydrolyzes lecithin to form phosphorylcholine. If suitable quantities of phosphorylcholine exist in a consumed food, intestinal upsets will become evident after 8 to 12 hours.

Since reconstituted NFDM, the substrate used in production of the protein hydrolysates, is essentially free of lecithin, it would seem improbable that phosphorylcholine could be present in the product.

Evaluation of Protein Hydrolysate

General Characteristics. As previously indicated, the general flavor characteristics of the proteolysate produced by B. cereus were similar to those produced by Ps. fluorescens as reported by Claydon (7) and Mickelsen et al. (23). Although the flavor tended to be bitter and somewhat objectionable in the early stages of production, the final product had a broth-like flavor and odor.

Effect of temperature. This study was made to determine the effect of incubation temperatures on the rate of proteolysis and to subsequently evaluate the flavor characteristics of the resulting product. Within the temperature limits of this experiment, B. cereus hydrolyzed the milk proteins at an increasing rate with increasing temperature as shown in Figure 2. Although the

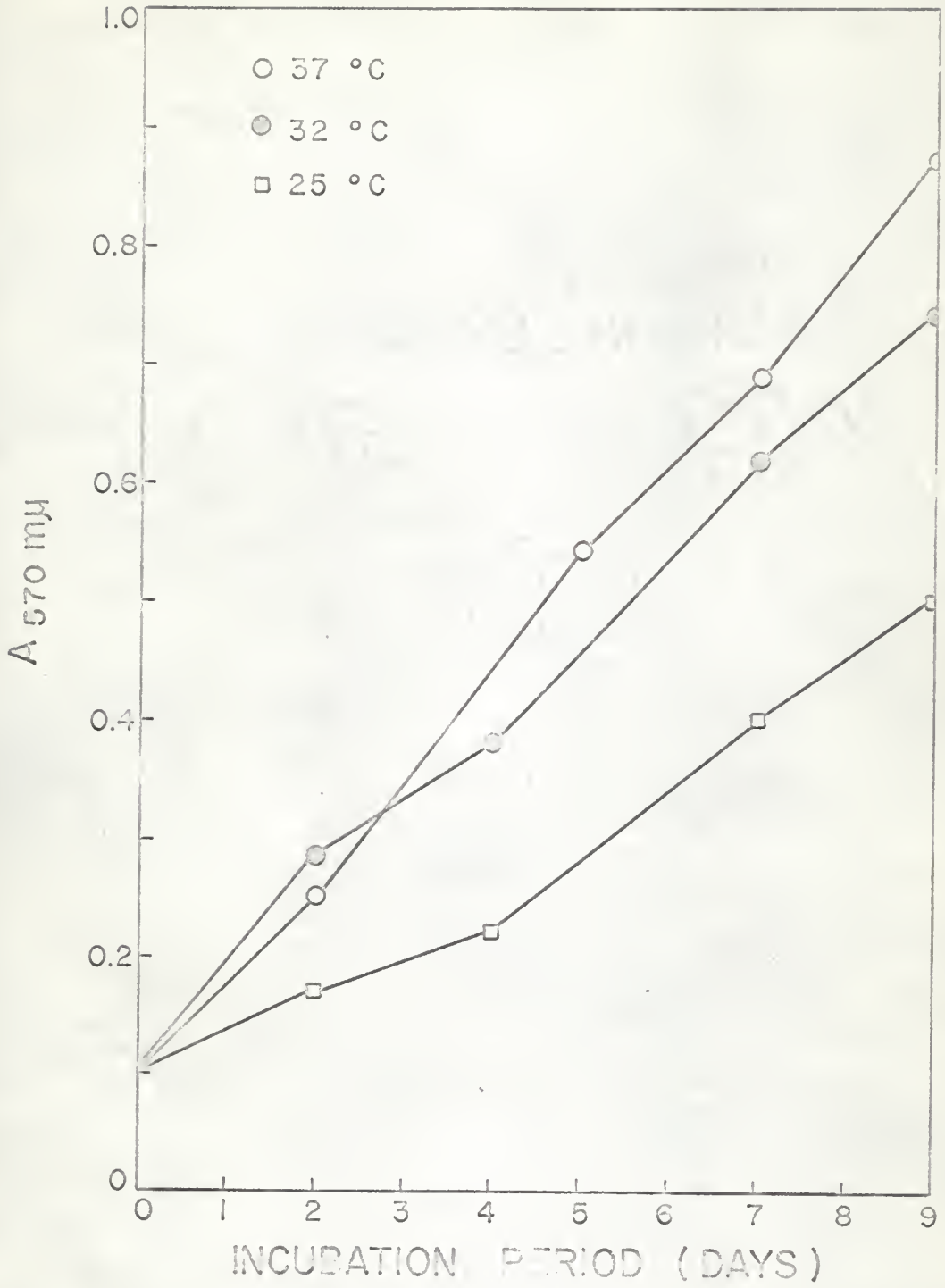


Figure 2. Effect of temperature on the rate of proteolysis of reconstituted NFDM by B. cereus.

milk was more extensively proteolyzed at 37 C than it was at 32 or 25 C, the differences were not great particularly between the two higher temperatures.

The proteolysate produced at each temperature was processed and evaluated for flavor and odor. The product resulting from hydrolysis at 25 C had the typical beef broth-like flavor. It was noted, however, that the products produced at 32 and 37 C had a slightly undesirable, unclean flavor. Since the incubation period was 9 days rather than the usual 7 days in this experiment, it was assumed that additional incubation at the higher temperatures may have resulted in the production of the unclean flavor. Some support for this assumption was provided by results obtained in a related experiment. In this study, five flasks of sterile reconstituted NFDM inoculated with B. cereus were incubated at 37 C with daily increments of time from 3 days to 7 days. The proteolysates were then processed and evaluated for flavor. The products resulting from the short periods of proteolysis were lacking in the characteristic flavor, compared to the products produced in the 5 to 7 day period. The latter had a desirable beef broth-like flavor and no undesirable flavors were noted.

Preliminary chemical and flavor characteristics. The proteolysate at each of the three main processing stages was fractionated by use of Sephadex G-25 and some chemical and flavor characteristics of the fractions were determined. Figure 3 shows the absorbancy at 280 mμ of the effluent from the Sephadex column at each processing stage. The absorbancy patterns were similar at each stage except for the larger peaks in the processed concentrate. The first peak, which came through the column immediately after the void volume, was presumably unhydrolyzed protein and the other peaks represented various protein degradation products.

The individual 5-ml fractions were evaluated organoleptically and found

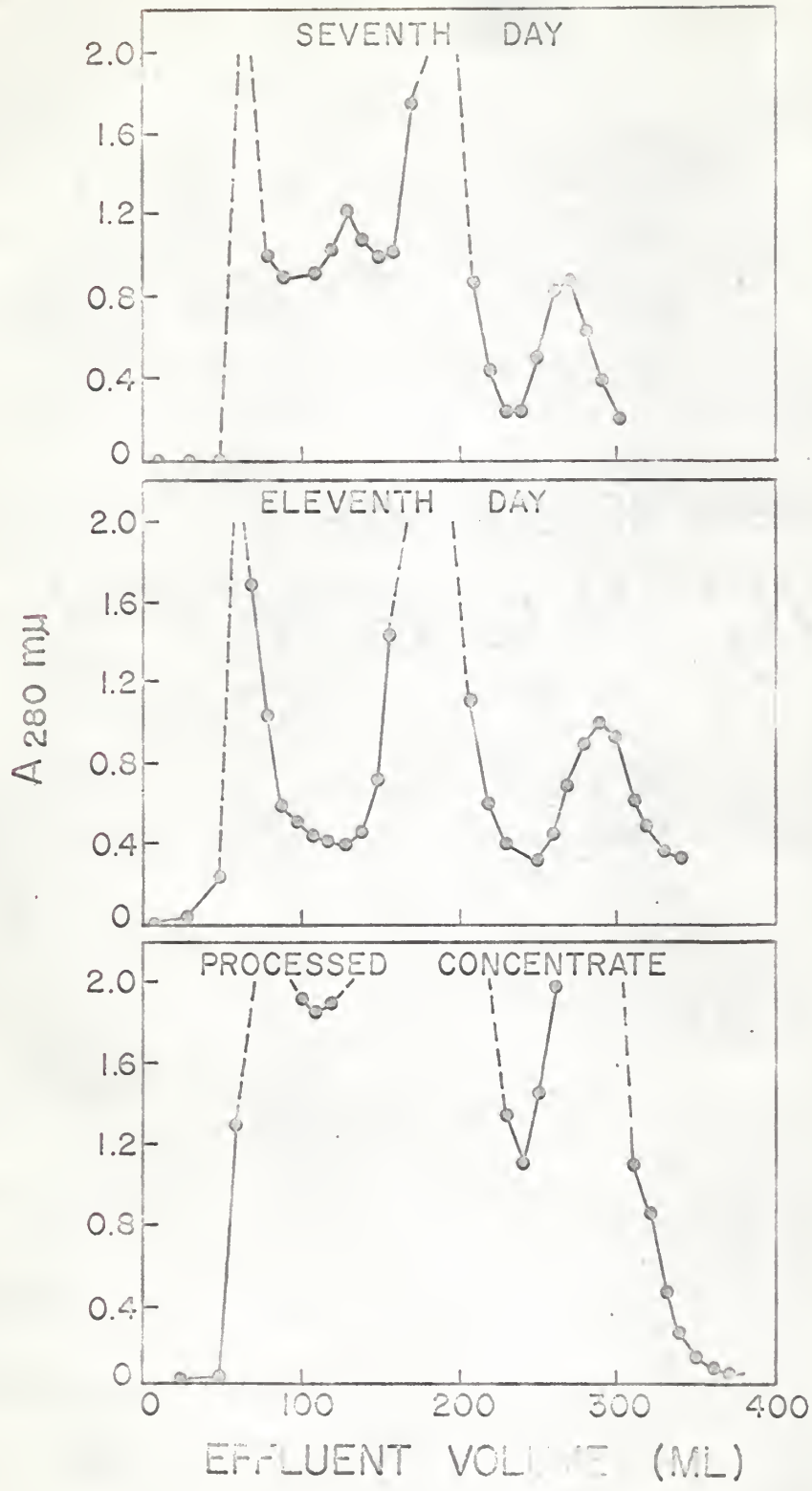


Figure 3. Absorbancy patterns at 280 mμ of the proteolysate fractionated on Sephadex G-25 at three main processing stages.

to vary from bitter and undesirable to a beef broth-like flavor. The characteristic brothy flavor was concentrated in the effluent from the 145-ml volume to the 160-ml volume in 7- and 11-day proteolysates, and in the 145-ml volume to the 175-ml volume in the processed concentrate. However, these brothy flavor fractions did not correspond to any of the peak maxima.

The absorbancy patterns and the flavor characteristics of the fractions at all stages of processing were similar to those produced by the proteolysate of Ps. fluorescens as reported by Mickelsen et al. (23). This may be an indication that B. cereus and Ps. fluorescens hydrolyzed the milk proteins in a similar manner.

Enzymatic Studies

Enzyme production. As discussed in the experimental procedures, reconstituted NFDM was found to be unsuitable for production of proteolytic enzymes by B. cereus mainly because of the small amount of enzyme produced in this medium. It was found, however, that a comparatively large amount of enzyme could be produced in Cheddar cheese whey. The difference in the amounts of enzyme produced in milk and in the whey over a period of seven days is shown in Figure 4. Approximately five times as much enzyme activity was present in the whey medium as in the milk. The amount of enzyme produced in both media apparently did not reach a maximum even at the end of seven days.

Analyses for the extent of proteolysis in both media were made simultaneously with the enzyme assays. Samples of whey and milk media were diluted 1:100 and 1:200, respectively, and the relative amount of proteolysis was determined by the ninhydrin method. Figure 5 shows the results of these analyses made over a period of seven days. It was noted that the whey substrate was not as readily

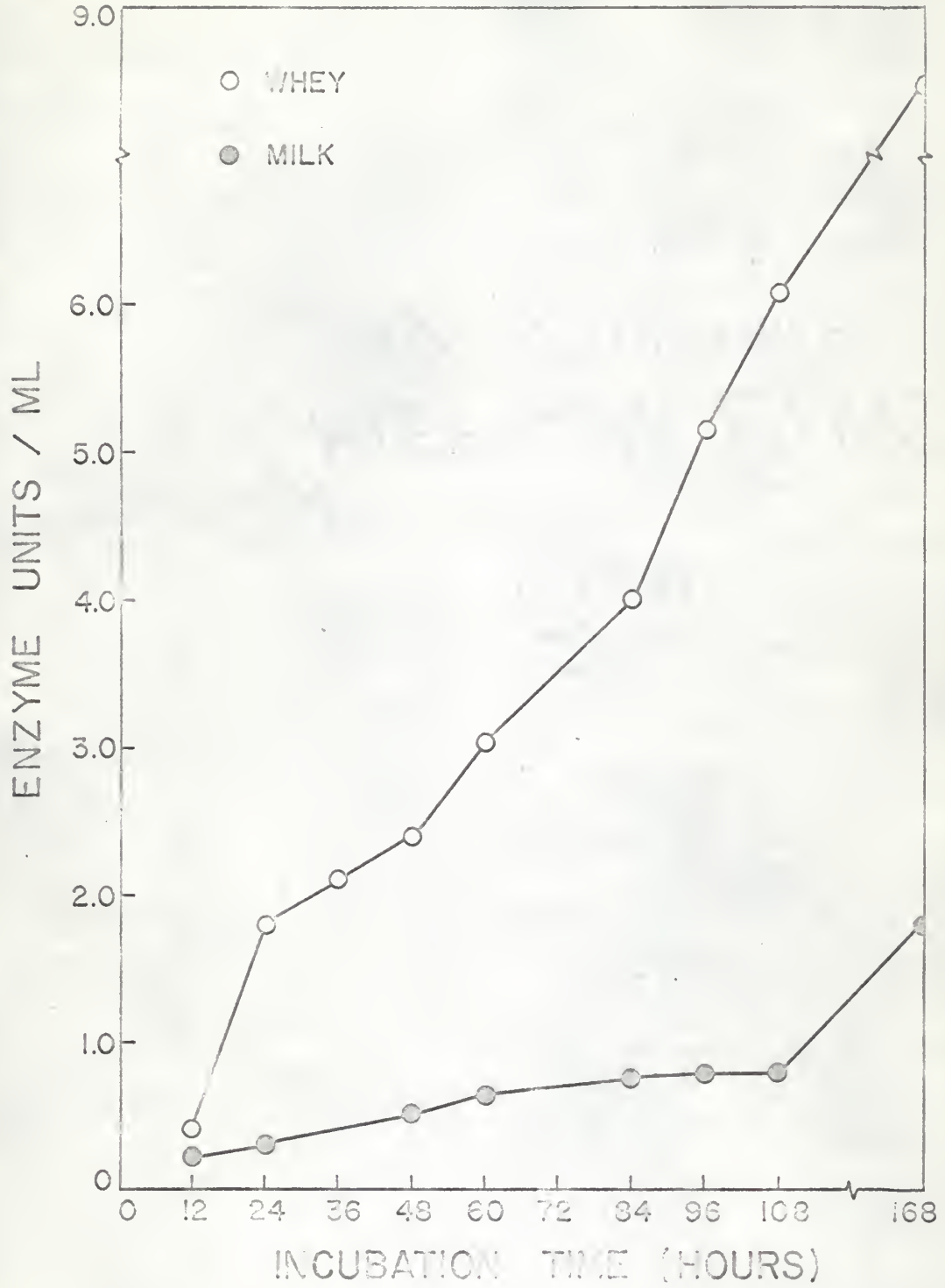


Figure 4. Amount of proteolytic enzyme produced by B. cereus in whey and in 10% solids reconstituted NFDM.

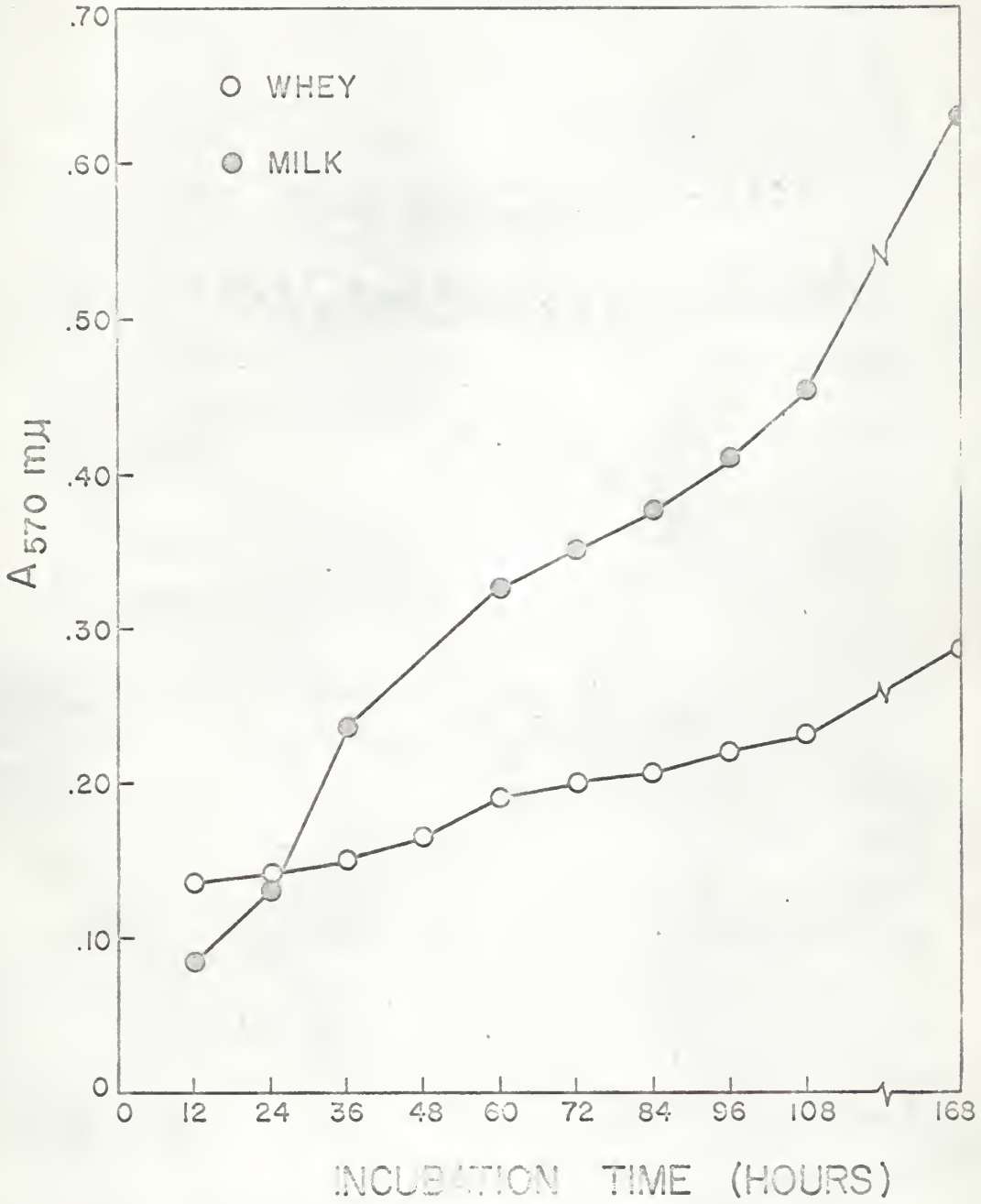


Figure 5. Relative amount of proteolysis produced by B. cereus in whey and in 10% solids reconstituted NFDM.

proteolyzed as the milk substrate, although the amount of proteolytic enzyme present in the whey was much greater. The small increase in proteolysis of the whey substrate indicated that the enzyme did not actively attack the whey proteins. On the other hand, the extensive proteolysis of the milk substrate indicated that casein was readily hydrolyzed with the liberation of high levels of amino acids. It has been reported that high levels of certain amino acids can repress the proteolytic enzyme production in B. cereus (18, 25). Consequently, this may be the reason why a comparatively small amount of proteolytic enzyme was produced in milk in comparison to that produced in whey.

Enzyme fractionation. The specific activity of the proteolytic enzyme was increased by precipitation with ethanol and by fractionation with ammonium sulfate. The ethanol precipitation, following centrifugation of the whey medium, was primarily a concentration step although a slight increase in specific activity was noted. Ammonium sulfate was added to the resuspended ethanol precipitate to 25% saturation. This resulted in the precipitation of some extraneous proteins, which then were centrifuged and filtered out, with a resulting increase in specific activity. The final step of the fractionation procedures was the addition of ammonium sulfate to 75% saturation which precipitated the proteolytic enzyme with a significant increase in specific activity. Table 2 illustrates the results of a typical fractionation. The procedures resulted in a 37-fold increase in specific activity with an over-all yield of approximately 20%.

Effect of temperature on enzyme activity. The enzyme preparation was incubated with casein substrate at eight different temperatures and the amount of proteolytic activity was determined at each temperature. The results, expressed as absorbancy at 280 mμ, are shown in Figure 6. Activity of the proteolytic enzyme increased with increasing temperature to 50 C. Temperatures

Table 2. Fractionation of the proteolytic enzyme from B. cereus.

Preparation	Vol. (ml)	Conc. (units/ml)	Total Units	Protein (mg/ml)	Specific Activity[1] (units/ml)	Yield %	Purification
Whey medium	1680	5.34	8971	.90	5.9	100	1
Ethanol precipitate	250	31.28	7820	2.75	11.4	87	2
25% saturation ammonium sulfate--supernatant	238	20.92	4979	.45	46.5	55	8
75% saturation ammonium sulfate--precipitate	54	32.64	1762	.15	217.6	20	37

[1]Specific activity = Concentration (units/ml) ÷ Protein (mg/ml)

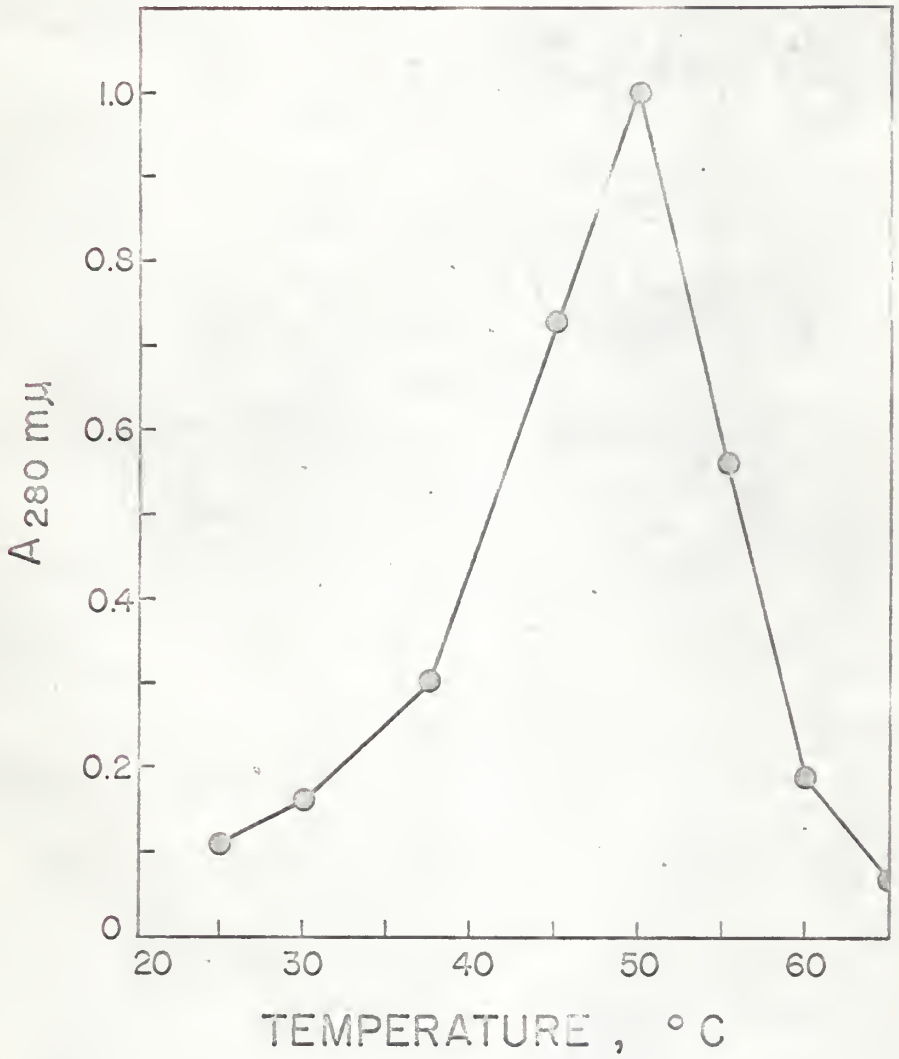


Figure 6. Effect of temperature on the activity of a proteolytic enzyme from B. cereus.

above 50 C reduced the activity of the enzyme with almost complete inactivation occurring at 65 C. The optimum temperature for activity of this enzyme from B. cereus was 50 C which is the same as reported by Grossman (13) for B. cereus strain number 7.

Effect of pH on enzyme activity. The optimum pH for proteolytic activity of the enzyme was determined by assaying it at eight pH values ranging from pH 5.0 to 9.0. Figure 7 shows the results of the assays with the amount of proteolytic activity expressed as absorbancy at 280 mμ. The amount of proteolytic activity at pH 5.0 was negligible, but increasing amounts of proteolytic activity were noted up to pH 7.5 with maximum activity occurring at this pH. This optimum pH value agrees with that reported by Grossman (13) for the proteolytic enzymes from three strains of B. cereus. Salter (29), however, reported that proteolytic enzymes from B. cereus NCTC 945 showed maximum activity at pH 6.8.

Effect of substrate concentration. The activity of the proteolytic enzyme on six concentrations of three different proteins was determined. As shown in Figure 8, the rate of proteolytic activity increased up to a protein concentration of 20 mg/ml in all three substrates. The most significant aspect of these results, however, is the observation that the enzyme did not hydrolyze the whey proteins, β-lactoglobulin and α-lactalbumin, as readily as it did the casein. It was found that β-lactoglobulin at a concentration of 20 mg/ml was hydrolyzed only 27% as much as casein at the same concentration. The α-lactalbumin was particularly resistant to hydrolysis by the enzyme. At the 20 mg/ml level of concentration, α-lactalbumin was hydrolyzed only 7.5% as much as casein. These observations of the relative inactivity of the enzyme on the whey proteins lends support to earlier results which showed that only a small increase in proteolysis

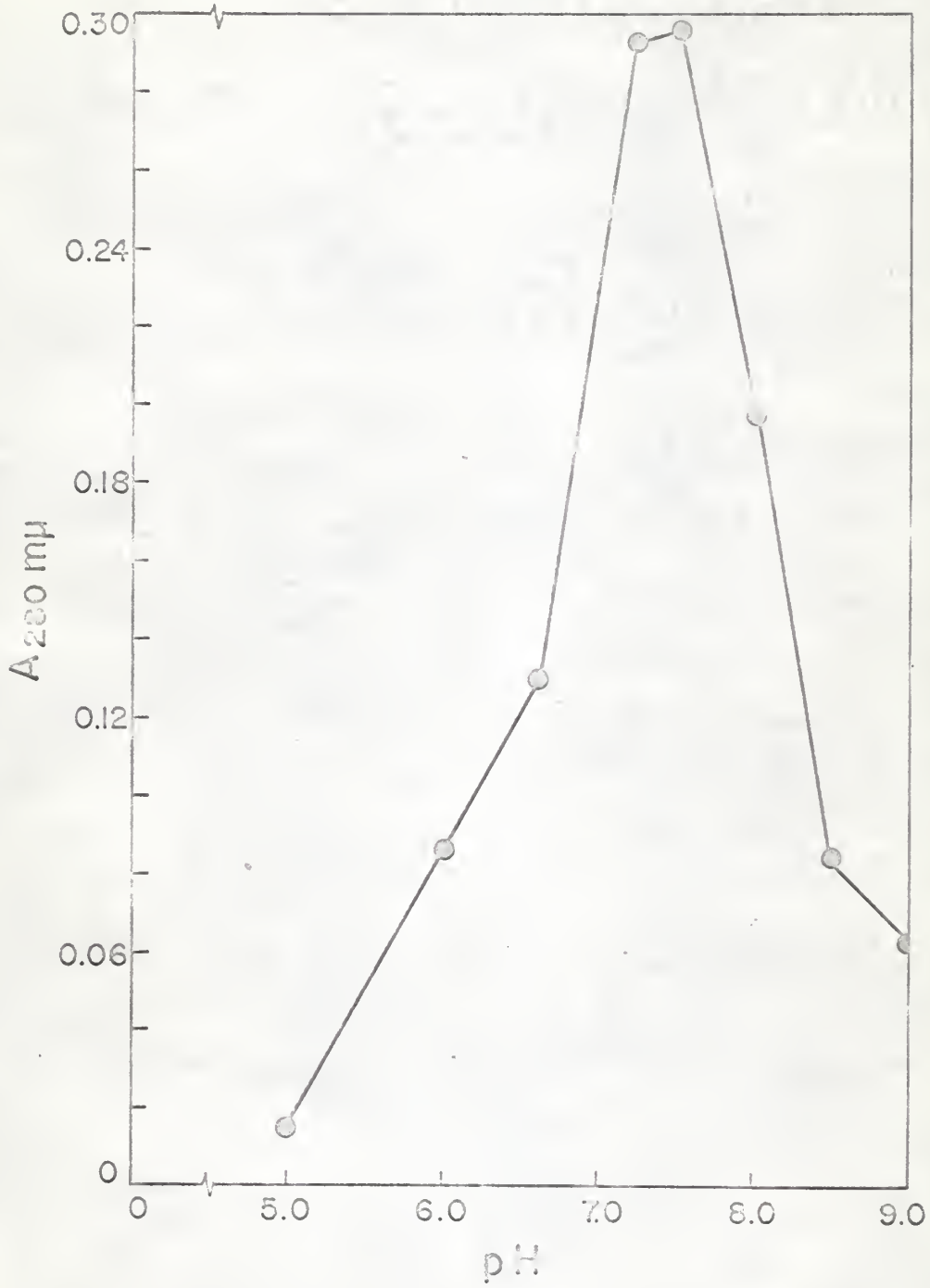


Figure 7. Effect of pH on the activity of a proteolytic enzyme from B. cereus.

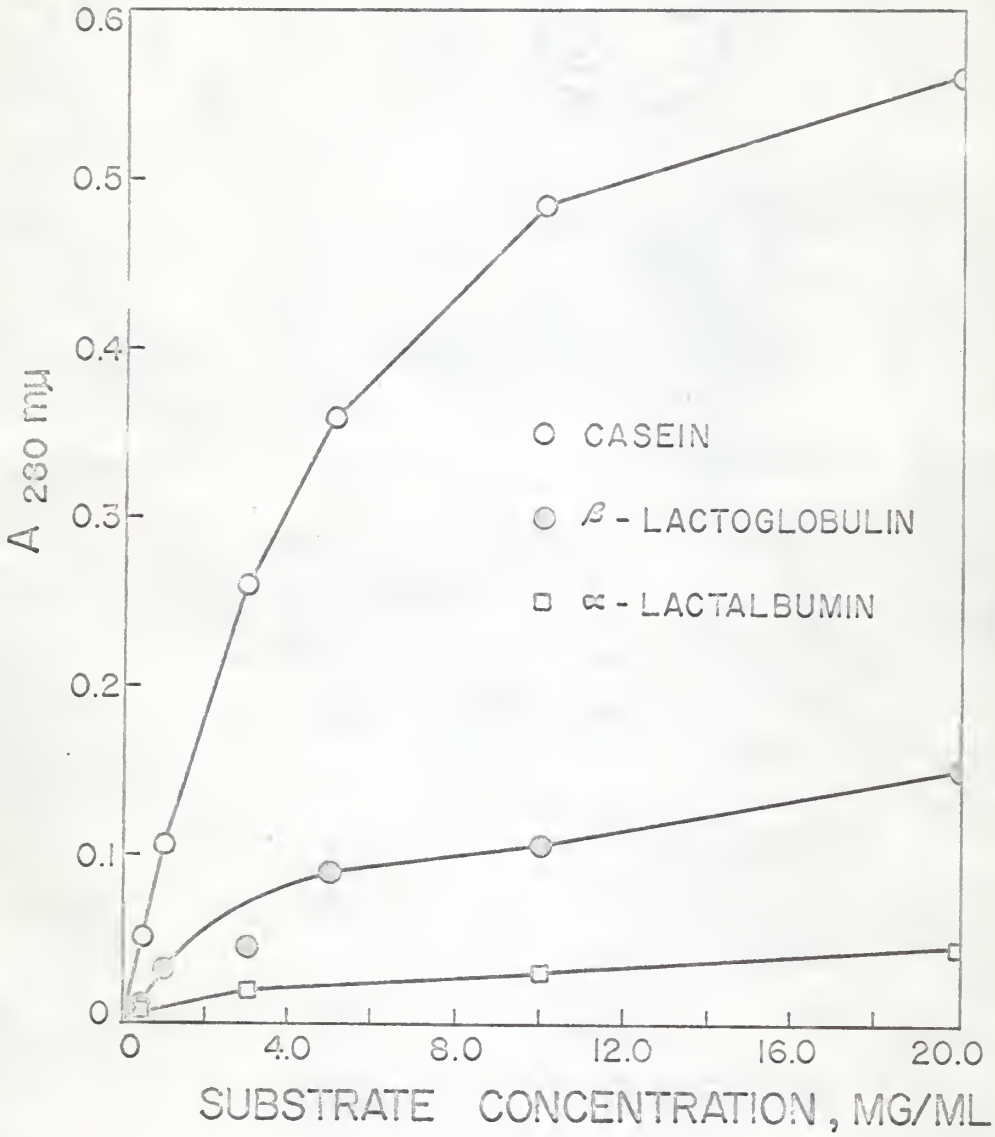


Figure 8. Effect of substrate concentration on the amount of proteolysis produced by a proteolytic enzyme from B. cereus.

occurred during the production of the proteolytic enzyme in whey.

A related study was made to determine the effect of the enzyme on hemoglobin at a concentration of 20 mg/ml. At this concentration, about five times as much casein was hydrolyzed as hemoglobin.

Effect of activators and inhibitors. Table 3 shows the effect of certain chemical compounds on the activity of the proteolytic enzyme. The activity of the enzyme in the presence of the individual compounds was compared to that of a control in which none of the chemicals was present.

The results indicate that $CaCl_2$ and $FeSO_4$ were the most stimulatory to the activity of the proteolytic enzyme. Two other compounds, $MgSO_4$ and $ZnSO_4$, also appeared to stimulate proteolytic activity, but to a lesser extent than $CaCl_2$ and $FeSO_4$. The remaining compounds tested were either inhibitory or had no effect on the activity of the enzyme. Of the various metal ions tested, only Cu^{++} and Mn^{++} were inhibitory, causing a 90% reduction in the amount of proteolytic activity. Almost complete inhibition by EDTA suggested that the enzyme required a metal ion for activity. Potassium oxalate, which specifically complexes with calcium and magnesium, reduced the activity of the enzyme to 76% of that of the control. The presence of iodoacetamide, a reagent that reacts with sulfhydryl groups, had no effect on the action of the proteolytic enzyme, thus indicating that sulfhydryl groups were not essential for activity. A reducing agent, cysteine, inhibited 47% of the proteolytic activity of the enzyme. One of the most effective inhibitors in this study was DFP. This reagent, which reacts with the hydroxyl group of serine, reduced the amount of activity to 5% of that of the control. Inhibition by this reagent may indicate that serine is a part of the active site of the enzyme. In this respect, the proteolytic enzyme from B. cereus would be similar to chymotrypsin, trypsin, subtilisin, and certain other enzymes (33).

Table 3. Effect of various compounds on the activity of the proteolytic enzyme from B. cereus.

Compounds[1]	Percent of control activity	Compounds	Percent of control activity
$CaCl_2$	138	EDTA	11
$CuCl_2$	11	Potassium oxalate	76
$FeSO_4$	151	Cysteine	53
$MgSO_4$	114	Iodoacetamide	102
$MnSO_4$	10	DFP	5
$ZnSO_4$	118		

[1]Concentration of DFP was 0.005 M; others were 0.01 M.

CONCLUSIONS

1. Of various proteolytic bacteria tested, B. cereus was highly proteolytic and seemed suitable for production of milk protein hydrolysates.

2. After processing, the milk protein hydrolysate had desirable, beef broth-like flavor characteristics.

3. Chemical and flavor characteristics of the fractionated proteolysate were similar to those produced by the milk proteolysate of Ps. fluorescens.

4. Approximately five times as much proteolytic enzyme was produced in Cheddar cheese whey as in reconstituted NFDM, but the milk was more extensively proteolyzed than the whey.

5. A 37-fold increase in the specific activity of the enzyme preparation was achieved by precipitation with ethanol and fractionation with ammonium sulfate.

6. Maximum activity of the proteolytic enzyme occurred at 50 C and at pH 7.5.

7. The proteolytic enzyme hydrolyzed casein more readily than it did β-lactoglobulin and α-lactalbumin.

8. Certain metallic compounds were stimulatory to enzyme activity, but other metal ions, chelating agents, and DFP were inhibitory.

ACKNOWLEDGEMENTS

The author wishes to express his sincere appreciation to Professor Ross Mickelsen, Department of Dairy and Poultry Science, for his helpful suggestions and advice during this investigation and for his assistance and guidance in the preparation of this thesis.

Appreciation is also extended to Dr. T. J. Claydon, Department of Dairy and Poultry Science, for his valuable suggestions during the period of research.

Acknowledgement is extended to Dr. C. L. Norton, Head, Department of Dairy and Poultry Science, and Dr. M. MacMasters, Department of Grain Science and Industry, for their review of this manuscript.

The author wishes to thank the Dairy and Food Industry Supply Association for providing a fellowship for part of the financial assistance during this investigation.

Grateful acknowledgement is extended to the staff members and graduate students of the Department of Dairy and Poultry Science for their advice and cooperation.

Sincere appreciation is expressed to my wife, Karen, for her understanding and encouragement during the course of this study and for her assistance in the preparation of this thesis.

LITERATURE CITED

1. Ansen, M. L. 1938. Estimation of Pepsin, Trypsin, Papain, and Cathepsin with Hemoglobin. J. Gen. Physiol., 22:79-89.

2. Babbar, I. J., Sprinivasan, R. A., and Chakravorty, S. C. 1964. Occurence of Extracellular Acidic Endopeptidase of an Aerobic Spore-Forming Bacterium. J. Dairy Sci., 47:922.

3. Bernlohr, R. W. 1964. Postlogarithmic Phase Metabolism of Sporulating Microorganisms. I. Protease of Bacillus licheniformis. J. Biol. Chem., 239:538-543.

4. Breed, R. S., Murray, E. G. D., and Smith, N. R. 1957. Bergey's Manual of Determinative Bacteriology. 7th edition. Williams and Wilkins, Baltimore, Maryland.

5. Camp, B. J. and Van Der Zant, W. C. 1957. Proteolytic Enzymes from Pseudomonas putrefaciens. II. Characteristics of an Endocellular Proteolytic Enzyme System. Food Res., 22:158-163.

6. Carr, J. W., Loughheed, T. C., and Baker, B. E. 1956. Studies on Protein Hydrolysis. IV. Further Observations on the Taste of Enzymic Protein Hydrolysates. J. Sci. Food Agr., 7:629-637.

7. Claydon, T. J. 1962. New Products from Nonfat Milk Solids. Manufactured Milk Products J., 53 (8):12-13.

8. Connell, J. E. 1966. Hydrolyzed Proteins in Modern Food. Canadian Food Industry., 37 (2):23-24, 26, 28.

9. Dack, G. M., Sugiyama, H., Owens, F. J., and Kirsner, J. B. 1954. Failure to Produce Illness in Human Volunteers Fed Bacillus cereus and Clostridium perfringens. J. Infect. Dis., 94:34-38.

10. Damodaran, M., Govindarajan, V. S., and Subramanian, S. S. 1955. Proteolytic System of Bacillus licheniformis. Biochim. Biophys. Acta., 17:99-110.

11. Elving, P. J., Markowitz, J. M., and Rosenthal, I. 1956. Preparation of Buffer Systems of Constant Ionic Strength. Anal. Chem., 28:1179-1180.

12. Emanuiloff, I. 1959. Proteolytic Enzymes from Bacillus mesentericus and Their Properties. Enzymologia, 20:173-182.

13. Grossman, R. M. 1959. The Isolation and Characterization of Extracellular Proteolytic Enzymes of Selected Species of the Genus Bacillus. Ph.D. Thesis. Kansas State University, Manhattan.

14. Hall, F. F., Kunkel, H. O., and Prescott, J. M. 1966. Multiple Proteolytic Enzymes of Bacillus licheniformis. Arch. Biochem. Biophys., 114:145-153.

15. Hall, L. A. 1946. Protein Hydrolysates-Flavor Ingredients for Foods. Food Industries, 18:681-684.

16. Hauge, S. 1955. Food Poisoning Caused by Aerobic Spore-forming Bacilli. J. Appl. Bact., 18:591-595.

17. Harley, W. C., Gardner, F. A., and Vanderzant, C. 1963. Some Characteristics of a Proteolytic Enzyme System of Pseudomonas fluorescens. J. Food Sci., 28:47-54.

18. Levisohn, S., and Aronson, A. I. 1967. Regulation of Extracellular Protease Production in Bacillus cereus. J. Bacteriol., 93:1023-1030.

19. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. 1951. Protein Measurement with the Folin Phenol Reagent. J. Biol. Chem., 193:265-275.

20. Malkames, J. P., and Walter, H. E. 1951. Food Uses Devised for Protein of Cheese Whey. Butter, Cheese, Milk Products J., 42 (9):26-27.

21. McConn, J. D., Tsuru, D., and Yasunobu, K. T. 1964. Bacillus subtilis Neutral Proteinase. I. A Zinc Enzyme of High Specific Activity. J. Biol. Chem., 239:3706-3715.

22. Melachouris, N. P., and Tuckey, S. L. 1967. Properties of a Milk-Clotting Microbial Enzyme. (Abstract). J. Dairy Sci., 50:943.

23. Mickelsen, R., Fish, N. L., and Claydon, T. J. 1967. Some Chemical and Flavor Characteristics of a Milk Proteolysate of Pseudomonas fluorescens. J. Dairy Sci., 50:172-176.

24. Murray, T. K., and Baker, B. E. 1952. Studies on Protein Hydrolysis. I. Preliminary Observations on the Taste of Enzymic Protein Hydrolysates. J. Sci. Food Agr., 3:470-475.

25. Neumark, R., and Citri, N. 1962. Repression of Protease Formation in Bacillus cereus. Biochim. Biophys. Acta., 59:749-751.

26. Nygren, B. 1962. Phospholipase C-Producing Bacteria and Food Poisoning. Acta. Path. Microbiol. Scand. Suppl. No. 160.

27. O'Brien, R. T., and Campbell, L. L., Jr. 1957. Purification and Properties of a Proteolytic Enzyme from Bacillus stearothermophilus. Arch. Biochem. Biophys., 70:432-441.

28. Peterson, A. C., and Gunderson, M. F. 1960. Some Characteristics of Proteolytic Enzymes from Pseudomonas fluorescens. Applied Micro., 8:98-104.

29. Salter, D. N. 1959. Purification and Properties of a Proteinase from Bacillus cereus NCTC 945. Biochem. J., 72:23p.

30. Smith, N. R., Gordon, R. T., and Clark, F. E. 1952. "Aerobic Sporeforming Bacteria". Agr. Monograph No. 16, Pg. 40. USDA, Washington, D. C.

31. Srinivasan, R. A., Anatharamaiah, S. N., Keshavamurthy, N., Anantakrishnan, C. P., and Iya, K. K. 1962. Preparation of Cheddar Cheese by Using Bacterial Enzyme. XVIth Intern. Dairy Congr. Copenhagen., Sec. IV:1506.

32. Van Der Zant, W. C. 1957. Proteolytic Enzymes from Pseudomonas putrefaciens. I. Characteristics of an Extracellular Proteolytic Enzyme System. Food Res., 22:151-157.

33. White, A., Handler, P., and Smith, E. L. 1964. Principles of Biochemistry. p. 249. McGraw-Hill Book Co., New York.

34. Zeimba, J. V. 1967. "Tailored" Hydrolysates--How Made, How Used. Food Engineering, 39 (1):82-85.

PRODUCTION OF A PROTEIN HYDROLYSATE BY FERMENTATION OF MILK USING *BACILLUS CEREUS* WITH A STUDY OF THE PROTEOLYTIC ENZYME INVOLVED

by

DENNIS HUGH COLE

B. S., University of Missouri, 1966

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Dairy and Poultry Science

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1968

The purpose of this study was to: (a) choose a proteolytic microorganism for use in fermentation of milk to produce a protein hydrolysate for food uses, (b) produce and evaluate the characteristics of the protein hydrolysate, (c) investigate methods of producing, purifying, and determining characteristics of the proteolytic enzyme.

From among several cultures of proteolytic bacteria, Bacillus cereus was found to hydrolyze milk proteins at a rapid rate and after further processing the resulting protein hydrolysate had a desirable, beef broth-like flavor. In comparison to Pseudomonas fluorescens, used in earlier work, B. cereus produced a similar product at a faster rate.

The proteolysate was produced by incubating flasks of reconstituted nonfat dry milk (NFDM) inoculated with B. cereus at 37 C for 7 days, after which it was inoculated with a lactic culture for fermentation of lactose and incubation was continued for 4 days at 37 C with daily neutralization of lactic acid. The final product was prepared by carbon treatment, flash evaporation, autoclaving, and concentration.

The most rapid proteolysis of milk took place at 37 C with smaller amounts of proteolysis occurring at 32 and 25 C. The flavor of the processed product after proteolysis at these temperatures was similar unless the incubation period exceeded 7 days. Incubation longer than 7 days at the two higher temperatures resulted in processed products with unclean flavors.

Characteristics of the proteolysate were determined at each main processing stage by fractionating the product on Sephadex G-25 and evaluating the fractions organoleptically and for absorbancy at 280 mμ. The absorbancy patterns were similar at each processing stage with the first peaks representing unhydrolyzed proteins and the later peaks representing protein degradation

products. Flavors of the fractions varied from bitter and undesirable to beef broth-like, but the characteristic brothy flavor fractions were not concentrated in any of the peak maxima. The proteolysate was similar in this regard to that produced by Ps. fluorescens fermentation.

Five times as much enzyme was produced in Cheddar cheese whey substrate as in reconstituted NFDM. However, the milk was more extensively proteolyzed than whey indicating that the whey proteins were resistant to hydrolysis by the proteolytic enzyme.

An increase in the specific activity of the enzyme preparations was achieved by concentrating the enzyme by ethanol precipitation and subsequently fractionating the precipitate with ammonium sulfate. A 37-fold increase in specific activity was achieved with an over-all yield of approximately 20%.

Activity of the enzyme increased with increasing temperature up to 50 C. Beyond this temperature proteolytic activity decreased and was almost completely inactivated at 65 C. The proteolytic enzyme was assayed at pH values ranging from pH 5.0 to 9.0. Activity increased to a maximum as the pH was increased to pH 7.5, but decreased at higher pH values.

The activity of the enzyme in three substrates increased up to a substrate protein concentration of 20 mg/ml. However, it was noted that casein was more readily hydrolyzed by the proteolytic enzyme than were the whey proteins, β-lactoglobulin and α-lactalbumin.

Activity of the proteolytic enzyme was stimulated by 0.01 M $FeSO_4$, $CaCl_2$, $MgSO_4$, and $ZnSO_4$, but inhibited by 0.01 M $CuCl_2$ and $MnSO_4$. Two chelating agents, ethylenediaminetetraacetate (EDTA) and potassium oxalate, also were inhibitory which indicated that certain metal ions were required for activity of the enzyme. Diisopropylfluorophosphate (DFP) reduced the activity of the enzyme to 5% of the control.