SOME FACTORS AFFECTING PROTEOLYSIS OF MILK BY *PSEUDOMONAS FLUORESCENS*

by

ROSA LUELLA THOMAS

B. S., West Virginia University, 1964

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

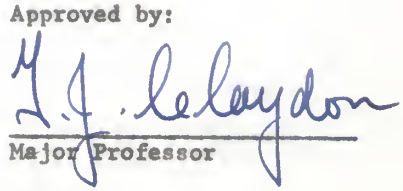
MASTER OF SCIENCE

Department of Dairy and Poultry Science

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1967

Approved by:



Major Professor

TABLE OF CONTENTS

INTRODUCTION

Protein hydrolysis (or proteolysis) consists of the breakdown of a native protein to simpler fractions such as proteoses, peptones, peptides and amino acids. It can be achieved with the use of acids, enzymes or alkali, but alkali is rarely used. In bacterial proteolysis, degradation of the substrate occurs as a result, initially, of the action of extracellular enzymes. In later stages, as cell autolysis occurs, proteolysis continues from both extra- and endoenzymes even in the absence of living cells. Consequently, conditions governing proteolysis are those affecting bacterial proliferation, enzyme elaboration, and enzyme activity.

When reconstituted nonfat dry milk (NFDM) is proteolyzed by Pseudomonas fluorescens and subsequently processed, a product with beef-extract characteristics can be produced (8). From a practical standpoint, one of the problems in the process is the length of time required for proteolysis. In addition, since the flavor of the final product is a prime consideration, any modification or acceleration of proteolysis must not produce end-products causing undesirable flavors.

The objective of the study reported in this thesis was to determine the conditions that would accelerate proteolysis of milk by Ps. fluorescens by a) accelerating bacterial development and enzyme action in growing cultures and b) extraction of the proteolytic enzyme system for direct use without living bacterial cultures. In addition, some attention had to be given to the potential effects on flavor of the final processed proteolysate.

LITERATURE REVIEW

The cultivation of microorganisms in media is dependent upon many nutritional and physical factors. Modification of some of these factors may

alter the metabolism in such a way that the production of specific metabolic products is either enhanced or inhibited. This review is limited to several factors favoring increased bacterial growth and proteolytic enzyme activity with emphasis on Pseudomonas species.

Effect of Temperature

Acceleration of bacterial proteolysis can be achieved by adjusting the temperature. Generally, most proteolytic enzymes, once elaborated, have their optimum activity at a rather high temperature. Van Der Zant and Moore (39), growing Pseudomonas species in skimmilk at four different temperatures (25, 21, 10 and 5 C), found that proteolysis was noticeable at 25 C and 21 C within 24 hrs but was negligible for the first five days at 10 and 5 C. They concluded that the more rapid proteolysis at higher temperatures might be due to either increased production or increased activity of the enzyme.

Peterson and Gunderson (31) isolated a Pseudomonas fluorescens culture from a frozen chicken pie which released the greatest amount of extracellular enzyme at low temperatures (0 C) even though its optimum growth temperature was 20 C. Once the enzymes were liberated, however, increases in temperature accelerated their activity greatly. The endocellular enzymes from cells grown at 10 C were three times as active per mg of enzyme protein as enzymes from cells grown at 35 C. In work by Hurley et al. (21), it was noted that enzyme preparations from Ps. fluorescens achieved maximum proteolytic activity at 37 C in a substrate of spray dried egg whites (SDEW) and at 25 C in hemoglobin.

Erickson and Evans (12) grew cultures, including Pseudomonas spp., in sterile whole milk at 5, 10, 15 and 20 C. It was observed that the rate of off-flavor production progressively increased as the temperature increased from 5 to 20 C. This was attributed to either an increased population of

cells and/or greater elaboration of enzymes which brought about milk decomposition.

Sandvik and Fossum (34) observed that Pseudomonas developed as the dominant group in whole milk upon long storage at 4 C. Greater amounts of proteinases developed at 10 and 20 C than at 4 C.

McDonald (28) studied three proteinases of Staphylococcus lactis that were most active at 45 to 55 C. Husain and McDonald (23) isolated an extracellular proteinase from Micrococcus freudenreichii that was most active at 50 C. Pavlasova and Starka (30) found proteases from Bacillus mycoides and Bacillus subtilis were most active at 46 C. An endocellular proteolytic enzyme system of Streptococcus lactis had its optimum activity against skimmilk and casein at 45 C (41). Baribo and Foster (4) worked with extracts (cells ground with glass) from Lactobacillus casei, S. lactis, and M. freudenreichii. The activity of the L. casei extract decreased rapidly above 37 C. The enzyme extract from S. lactis was most active at 40 and 42 C and the extract from M. freudenreichii had an optimum activity at 30 C. The peptidases from Pseudomonas putrefaciens showed optimum activity at 45 C (7). Van Der Zant (38), working with an extracellular proteolytic enzyme system from Ps. putrefaciens, observed its maximum activity against casein at 37.5 C, and against lactalbumin at 45 C. Decreases in activity took place at temperatures above 45 C.

Effect of pH

Bacterial proteolysis is affected by pH. The majority of bacterial proteolytic enzymes have a pH optimum above neutrality. Hurley et al. (21) found that enzyme preparations of Ps. fluorescens had an optimum activity at pH 7.5 in hemoglobin substrate and an optimum in SDEW between pH 7 to 9. Cultures of Ps. fluorescens adjusted to pH values of 6, 7 and 8 in nutrient

broth adjusted themselves to values ranging from 7.3 to 7.9.

Peterson and Gunderson (31) grew Ps. fluorescens in flasks of tryptone-glucose-meat extract broth having initial pH values between 5.5 and 9.0. Increments of 0.5 pH units were used. The pH of 8.0 was optimum for growth while pH 7.0 and 8.5 were optimum for the liberation of extracellular enzymes. The two separate pH optima suggested two different extracellular enzymes. The extracellular enzymes had peaks of proteolytic activity at pH 6.2, 7.8 and 8.8.

Pavlasova and Starka (30) found that the optimum pH of B. mycoides protease was 7.3 to 9.8 while that of B. subtilis was 7.3. All three proteinases of Staph. lactis had maximum activities at pH 9.0 to 10.0 (28). Camp and Van Der Zant (7) observed optimum hydrolysis for peptidases of Ps. putrefaciens between pH 7.0 and 8.0. The activity decreased at pH values below 6.0. The peptidase of S. lactis showed optimum activity at pH values between 7.0 and 8.5 (42).

Proteolytic enzymes having a pH optimum below neutrality are of importance in the cheese industry. Babbar et al. (3) found that the pH optima of enzymes from Bacillus spp. are neutral or alkaline to about pH 10. They did, however, find an endopeptidase from B. subtilis with an optimum of pH 5.5. The pH for maximum proteinase activity of the cell-free extract of L. casei was between pH 5.5 to 6.5 at 37 C (5). Husain and McDonald (23) isolated an extracellular proteinase from M. freudenreichii that was most active at pH 5.5 to 6.4. Vand Der Zant and Nelson (41) studied an endocellular proteolytic enzyme of S. lactis. Maximum activity against skimmilk and casein was found at pH 7.0 with some indication of a second optimum at pH 5.5.

Effect of Activators and Inhibitors

Kraft and Ayres (25) treated dressed chickens with a solution containing 10 µg $FeCl_3 \cdot 6H_2O$/ml and placed them in storage. Greater numbers of total aerobes and of fluorescing bacteria developed on the treated chickens than on those not treated with iron. Garibaldi and Bayne (16) studied the effect of iron on Pseudomonas spoilage of experimentally infected shell eggs. A solution containing 10 mg of $FeSO_4$/l was used for washing eggs. Almost all of the eggs became contaminated. Ps. fluorescens invaded the eggs with iron levels as low as 1.0 mg/l.

Hurley et al. (21) preincubated one ml of an enzyme preparation from Ps. fluorescens with one ml of 0.01, 0.005 and 0.001 M solutions of $FeCl_2$, $FeCl_3$, $CoCl_2$, $MnSO_4$ and cysteine. After the preincubation, the substrate (SDEW or hemoglobin) was added and the normal incubation was carried out. All of the concentrations of $FeCl_2$ activated proteolysis with the concentration of 0.01 M activating the most. Cysteine, $MnSO_4$ and $FeCl_3$ inhibited proteolysis at all concentrations. $CoCl_2$ inhibited proteolysis at concentration of 0.01 and 0.005 M but activated proteolysis at 0.001 M.

The hydrolysis of di- and tri-l-ornithine by ornithine peptidases from Bacillus spp. was accelerated by the presence of 0.001 M Mn^{++} and to a lesser extent by the same concentration of Co^{++} (13). Levinson and Sevag (26) showed that spores of Bacillus megaterium contained proteolytic enzymes and that the activity of these enzymes was accelerated by a solution of 10 ppm $MnSO_4$.

Cobalt (as 0.05 M $CoSO_4 \cdot 7H_2O$) had a marked effect on the enzymic hydrolysis of peptides by peptidases of L. casei. Manganese (as 0.05 M $MnSO_4 \cdot 4H_2O$) was tried but was inhibitory or had no effect at all (6).

The proteolytic activity of a proteinase from a Trichosporon species

with casein as substrate was activated slightly by 1 to 10 MM cysteine and 0.1 to 1 MM Co^{++}. There was slight inhibition by 10 MM Co^{++} (18). Cysteine exerted a slight stimulatory effect on the activity of the enzyme in a concentration of 5×10^{-3} M (43). Whitaker (44), working with proteolytic enzymes of commercial ficin, showed that 0.025 M cysteine completely activated 3.13×10^{-2} µg of ficin in one ml of 0.2 M buffer at pH 6.72 or 9.5. Activation was so rapid that a period of incubation was not necessary.

Not all enzymes are activated by metallic ions or reducing agents. Baribo and Foster (4) and Van Der Zant (38) found that cysteine, $FeCl_3 \cdot 6H_2O$, $FeSO_4 \cdot 7H_2O$, $Fe(SO_4)_3$, $MnSO_4 \cdot 4H_2O$, $CoCl_3$ and $CoSO_4 \cdot 7H_2O$ did not activate proteinases of certain organisms from cheese or the extracellular proteolytic enzymes from Ps. putrefaciens.

Effect of Aeration

Electrolysis is a relatively new method for aerating bacterial cultures. Sadoff et al. (33) achieved the same growth rate and the same final cell densities of Ps. fluorescens in electrolyzed cultures as in those cultures aerated by agitation. Graphite cathodes were inhibitory to bacterial growth and had to be replaced with iron. They also found that the cultures were inhibited at dissolved oxygen concentrations above 4.65 ml/l of glucose-mineral salts medium.

Agitation has been used in the production of enzymes from microorganisms (3, 18, 28, 37). Friedman et al. (14), however, found that although the growth of Bacillus linens was proportional to the amount of aeration, the amount of proteinase formed was not. Nashif and Nelson (29) showed that the shaking of the growth medium twice daily lowered both cell population and lipase production of Pseudomonas fragi. The greater surface/volume of

the liquid medium was found to give higher counts and greater lipase activities. Cordon and Schwartz (9), growing Beauveria tenella, and Peterson and Gunderson (31), working with proteolytic enzymes from Ps. fluorescens, favored shallow layers of the growth medium when growing their cultures. Hurley, et al. (21) added sterile air (1 psi) to growing cultures of Ps. fluorescens before characterizing its proteolytic enzyme system.

Methods for Enzyme Purification and Evaluation

Preparation of cell-free extracts. If extracellular enzymes are desired, the bacterial cells can be removed by centrifugation (3, 21, 45) and the supernatant then passed through a bacterial filter (31, 38). For endocellular enzymes, the cells are usually harvested by centrifugation, washed with a buffer and then treated by sonic vibration (7, 14, 18, 31, 40, 42) or ground by means of glass beads, silica or a Booth-Green Mill (4, 41) and removed by centrifugation before fractionating.

Fractionation procedures. Enzymes, as they become concentrated, are subject to loss of activity upon exposure to heat, organic solvents, extremes of pH, surface denaturation (foaming) and heavy metals (10, 11, 36). It is advisable to treat enzymes carefully and to avoid conditions in which they will become unstable.

Most fractionationsinvolve use of the salting out effect of $(NH_4)_2\ SO_4$ in at least one stage followed by dialysis (3, 14, 18, 21, 31, 38, 42, 45). Other methods include using absorbents such as kaolin and $Al(OH)_3$ (42), or calcium phosphate gel treatment (45) and solvent precipitation with acetone (15), methanol (17), or a 3:1 mixture of acetone and ethanol (24). Column chromatography using Sephadex G-100 (18) is employed as is Diethylaminoethyl cellulose (21, 45) and carboxy methyl cellulose (21). Starch gel electro-

phoresis (18) and lyophilization (3, 46) are also used.

Determination of proteolytic activity. A crude method for detecting proteolysis is to use solidified agar plates containing the substrate to be tested (such as milk or egg white), placing paper discs previously dipped into the culture or enzyme preparation upon the agar surface, and then incubating the agar plates. Plates are observed at certain intervals for zones of clearing or diameter of growth (22, 32).

McDonald (28) and Peterson and Gunderson (31) used the assay technique of Anson (2) for measuring amino acids freed by proteolytic enzymic action. Casein was used as the substrate and the amount of tyrosine and tryptophan that reacted with the Folin-Ciocalteau reagent was determined by light absorption at 645 mu.

The Hull method (20) to determine tyrosine and tryptophan by partial hydrolysis of milk protein was used by Van Der Zant and Nelson (40). Van Der Zant and Nelson (42), studying endocellular peptidases, determined the hydrolysis of the peptides by titration of the carboxyl groups with ethanolic KOH, using thymolphthalein as indicator.

Protein and nitrogen determination. Protein nitrogen values are generally determined by Kjeldahl methods (4, 21, 31, 38, 40).

Determination of protein breakdown can be made by the Folin Ciocalteau phenol method which detects tyrosine and tryptophan (27, 45) and by measurement of absorbancy of TCA filtrates at 280 mu in a Beckman DU Spectrophotometer (36). The ninhydrin reaction that measures alpha amino acids, the biuret reaction which detects the presence of two or more peptide linkages and the formol titration in which formaldehyde reacts only with the $-NH_2$ group of an amino acid are commonly used for protein degradation measurement (19).

EXPERIMENTAL PROCEDURES

The first part of the investigation involved the proteolysis of milk by Ps. fluorescens under various conditions. The latter part pertained to attempts to separate and concentrate the proteolytic enzyme system.

Fermentation Procedures

Culture propagation. Ps. fluorescens cultures were propagated in litmus milk. Stock cultures were transferred weekly, incubated at 25 C for 2 days and then stored in the refrigerator after development. For the proteolysis and enzyme studies, cultures that had been grown 2 days at 25 C prior to each trial were used as inoculum.

Media. Litmus milk used for culture propagation was prepared from 10% reconstituted nonfat dry milk (NFDM) and autoclaved at 15 psi for 15 minutes. Standard Plate Count agar was used for counting viable organisms by the plating method (1) and the plates were incubated at 25 C for 48 hours.

Milk agar plates were used for testing for the presence of viable organisms, culture purity, and in screening tests for proteolytic activity. Plates were poured from Standard Plate Count agar containing 5% sterile litmus milk added just before pouring. For measuring proteolytic activity, exactly 10 ml of the medium were used in flat bottom antibiotic testing plates.

Milk for proteolysis studies was reconstituted NFDM made to 10% solids. The milk was dispensed in 250 ml quantities into 1 liter Erlenmeyer flasks and in 500 ml quantities into 2 liter Erlenmeyer flasks, then autoclaved at 15 psi for 15 minutes. After cooling, the milk was stored at room temperature until used.

Production of proteolysate. One ml of Ps. fluorescens culture was inoculated into flasks of prepared milk. Flasks were incubated at the desired

temperature for the desired periods. The extent of proteolysis was followed visually and by chemical tests. This procedure was used for both bacterial growth and enzyme studies.

Effect of temperature. Inoculated flasks of milk were placed at temperatures of 25, 30, 37, 40 and 45 C. The pH was not adjusted in any way. Ninhydrin tests (a measure of alpha amino acids) were used throughout the experiments to indicate the rate and amount of proteolysis. Streak plates of Standard Plate Count agar containing a milk base were used to indicate bacterial viability.

Effect of pH. Flasks of inoculated milk were adjusted to pH values of 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 with either 2N HCl or 3N NaOH and incubated at 30 C. No adjustment of pH was made during the proteolysis.

Effect of activators and inhibitors. Flasks of inoculated 10% NFDM were prepared and the compound added at various concentrations. Three flasks were used for each compound. In those flasks containing $FeSO_4 \cdot 7H_2O$, one had a concentration of 20 mg/ml, the second a concentration of 40 mg/ml and the third had a concentration of 60 mg/ml. A similar procedure was used with the other compounds but with different concentrations, as follows: 1, 5 and 10 mg $FeCl_3 \cdot 6H_2O$/ml; 0.1, 0.01 and 0.001 M $MnSO_4 \cdot H_2O$/ml; 0.1, 0.01 and 0.001 M cysteine/ml; 0.1, 0.01 and 0.001 M $CoSO_4 \cdot 5H_2O$/ml. The control flasks contained no additives. Incubation was at 25 C for all of the flasks except those of $FeCl_3 \cdot 6H_2O$ and they were placed at 37 C after 5 days of initial growth at 25 C.

Methods of Analysis

Test for ninhydrin reactive compounds. This test was used as a measure of protein breakdown. One ml of diluted sample was placed in a test tube.

One ml of ninhydrin reagent (95 g KH_2PO_4, 43 g Na_2HPO_4, 5 g ninhydrin and 3 g fructose/1000 ml of water) was added to the test tube and the tube placed in a boiling water bath for 15 min. At the end of this time, the test tube was cooled in cold water, 5 ml of ninhydrin diluent (2 g KIO_3, 600 ml distilled water and 400 ml 95% ethanol) added and the solution read at 570 mu on the Spectronic 20. The blank was prepared by substituting one ml of distilled water for the sample and not boiling. The percent transmission reading was converted to optical density, the optical density was multiplied by the dilution and the resultant reading was expressed as the ninhydrin value. The dilution of the sample should be such that the percent transmission reading of the sample falls between 30 and 70.

Zones of proteolysis. Paper discs (6.5 mm in diameter) were made from Whatman #41 filter paper with a hole puncher. One ml of toluene was added to 1 ml of enzyme preparation (or proteolyzed milk) and mixed to an emulsion. A paper disc was dipped into the mixture and then placed on milk agar plates. Several discs could be used per plate. The plates were incubated at the desired temperature and the diameter of the clear proteolyzed zones measured at the end of 24 hrs.

Enzyme activity test. This was conducted according to the method of Hurley et al. (21). It measures tyrosine as a product of protein hydrolysis. One ml of the proteolyzed milk medium or enzyme preparation was added to 5 ml of substrate consisting of 1% NFDM in water. Following incubation for 60 min in a water bath at 37 C, 10 ml of 0.3 M trichloracetic acid (TCA) was added to terminate the reaction and to precipitate the remaining undigested protein. The tubes remained in the water bath for an additional 1 hr. The contents of the tubes were then filtered through Whatman #3 filter paper. Measurement of absorbancy of the TCA filtrate at 280 mu in a Beckman DU Spectrophotometer

was used as an index of tyrosine content and extent of protein hydrolysis. Blanks were run with each trial by the addition, first of 10 ml of 0.3 M TCA to 5 ml of 1% NFDM, and then the enzyme preparation was added. They remained at room temperature until filtered.

In order to relate absorbancy at 280 mu to tyrosine content, a standard curve was prepared from tyrosine solutions of various concentrations. A stock solution of tyrosine was prepared that contained 0.4 mg tyrosine/ml. In order to dissolve the tyrosine, the solution was acidified with concentrated HCl to a pH of 2.5 Dilutions of 0.3, 0.2 and 0.08 mg/ml were made from the stock solution of 0.4 mg/ml. These solutions were tested in the same manner as in the activity test except that there was no heating or incubation involved.

In determining nitrogen, the semi-micro Kjeldahl procedure of Shahani and Sommer (35) for total nitrogen was used. Modification of the procedure consisted of using 1 ml of sample directly without diluting. The formulas used for calculating specific activity, total activity and percent yield were:

$$\text{mg N/ml} = (\text{ml HCl} - \text{ml Blank}) \times \text{N of HCL} \times 14.008$$

$$\text{Specific Activity} = \frac{\text{mg tyrosine/ml}}{\text{mg N/ml}} \quad \text{(from tyrosine curve)}$$

$$\text{Total Activity} = \text{volume} \times \text{Specific Activity} \times \text{mg N/ml}$$

$$\% \text{ Yield} = \frac{\text{total activity of a given preparation}}{\text{total activity of the starting material}} \times 100$$

Enzyme Fractionation Procedures

One l of 7-day old proteolyzed milk was centrifuged for 30 min in a refrigerated centrifuge (0 C) at 3500 X g. After centrifugation, further fractionation steps were performed in an environment of 4 C. The precipitate, after centrifuging, was discarded and granular $(NH_4)_2SO_4$ was added to the

supernatant to make a 50% concentration. The mixture was allowed to stand for approximately 24 hrs and was then filtered through Whatman #3 filter paper. The precipitate was dissolved in a volume of phosphate buffer (pH 7.0, 0.01 M Na_2HPO_4 and NaH_2PO_4) equal to one tenth the volume of the original supernatant. The reconstituted precipitate was placed in seamless cellulose dialysis tubing (soaked in disodium ethylenediaminetetraacetate for one-half day then washed with several changes of distilled water before using). This was to remove any lead ions that may have remained in the tubing after manufacturing. Dialysis was against the pH 7.0 phosphate buffer (0.01 M) above or pH 7.5 Na_2HPO_4 and KH_2PO_4 buffer and pH 7.9 Na_2HPO_4, KH_2PO_4 buffer for approximately 24 hrs. Samples for Kjeldahl analysis and enzyme activity tests were taken at various treatment stages and frozen or lyophilized for future use.

Other fractionation techniques were also tested, such as pre-precipitation by acid and heat, different centrifugation speeds and varying the concentration of $(NH_4)_2SO_4$. The use of acetone, ethanol and methanol and combinations of precipitation techniques were tried. Column chromatography using Sephadex G-25 was also tried. Bacterial cells were disrupted with the French Pressure Cell to test for endoenzymes. The results from these procedures were inconsistent and no improvement was obtained over the $(NH_4)_2SO_4$ technique.

RESULTS AND DISCUSSION

Effect of Temperature

In preliminary trials, when inoculated flasks were placed immediately at the various incubation temperatures, it was evident that no growth of Ps. fluorescens or proteolysis occurred at 40 or 45 C. Consequently, in

subsequent trials, all flasks were incubated initially for 5 days at 25 C for bacterial development and enzyme elaboration.

After initial development, subsequent proteolysis proceeded more rapidly at the higher temperatures as shown in Figure 1. This is one of several trials. The highest ninhydrin values occurred at 40 C followed closely by those at 45 C. The incubation temperature of 25 C had the lowest ninhydrin values. The proteolysis at 45 and 40 C was different from that which occurred at 25 C. The higher temperature proteolysates became dark brown in color with a pungent odor. Apparently, different enzyme systems and end products were involved.

In view of the fact that this research was part of an overall project directed toward developing a possible beef-extract substitute, the undesirable odors obtained at the higher temperatures of 40 and 45 C offset the advantage of more rapid proteolysis. There was no viable growth of Ps. fluorescens in the flasks (as shown by streakings on milk agar plates) at 45 C after 3 days and at 40 C after 4 days. Hence, it is thought that the continued proteolysis was due to enzyme action. Growth continued at the other temperatures throughout the experiment.

Effect of pH

The adjustment of flasks of inoculated 10% NFDM to initial pH values above 7.0 did not necessarily bring about increased proteolysis. Results from one of several trials are shown in Figure 2. The flasks were initially adjusted to the desired pH and incubated at 30 C. No adjustment of pH was made during the proteolysis. Ninhydrin values were highest and quite similar for flasks with initial pH values of 6.5 and 7.0. The lowest ninhydrin values were at pH 9.0. The intermediate pH values of 8.0 and 8.5 also showed intermediate ninhydrin values that were quite similar to one another. The

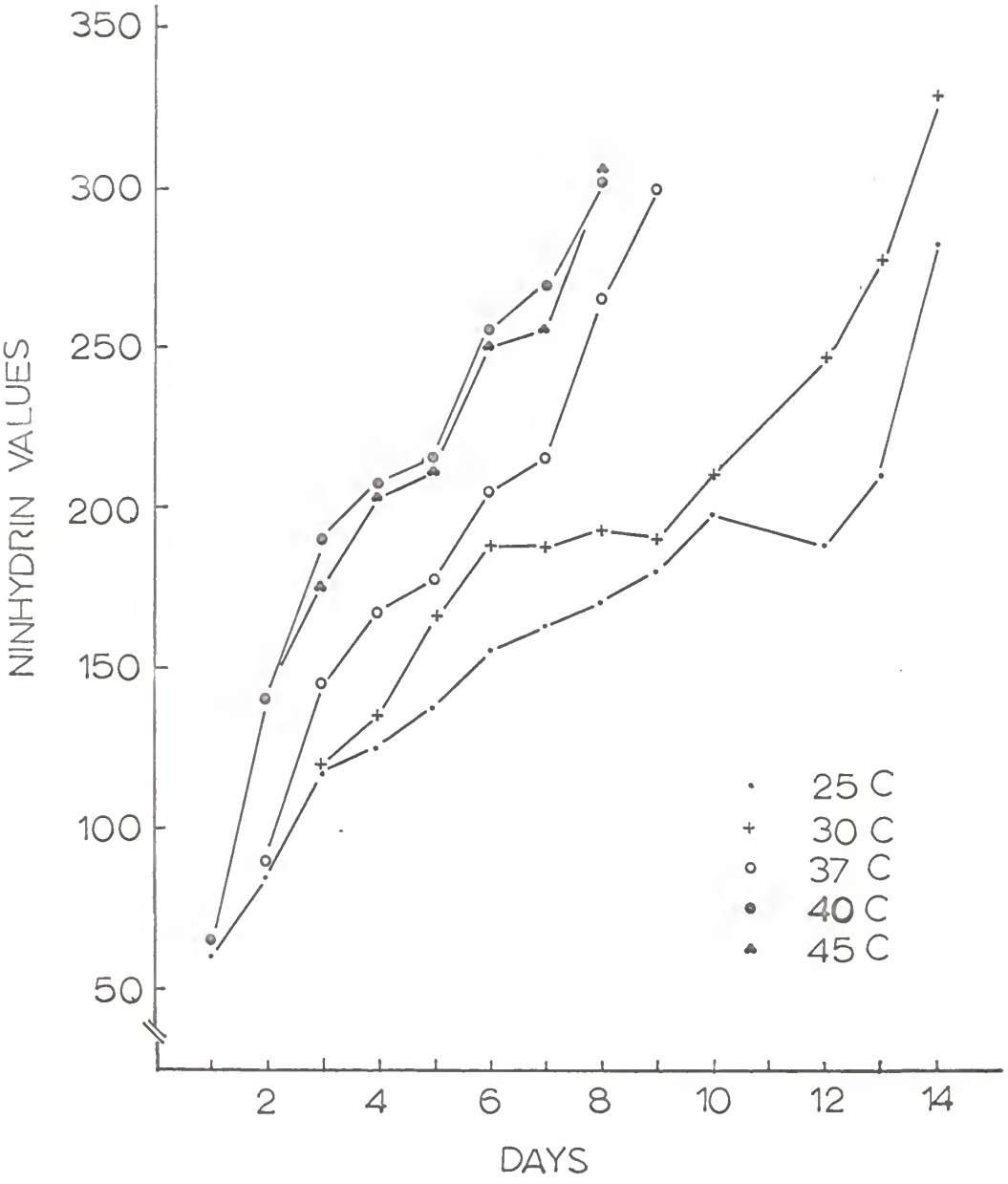


Fig.1. Effect of temperature on proteolysis of milk by Ps.fluorescens after 5 days initial growth at 25 C. Growth ceased after 3 days at 45 C, and after 4 days at 40 C but continued for 14 days at the other temperatures.

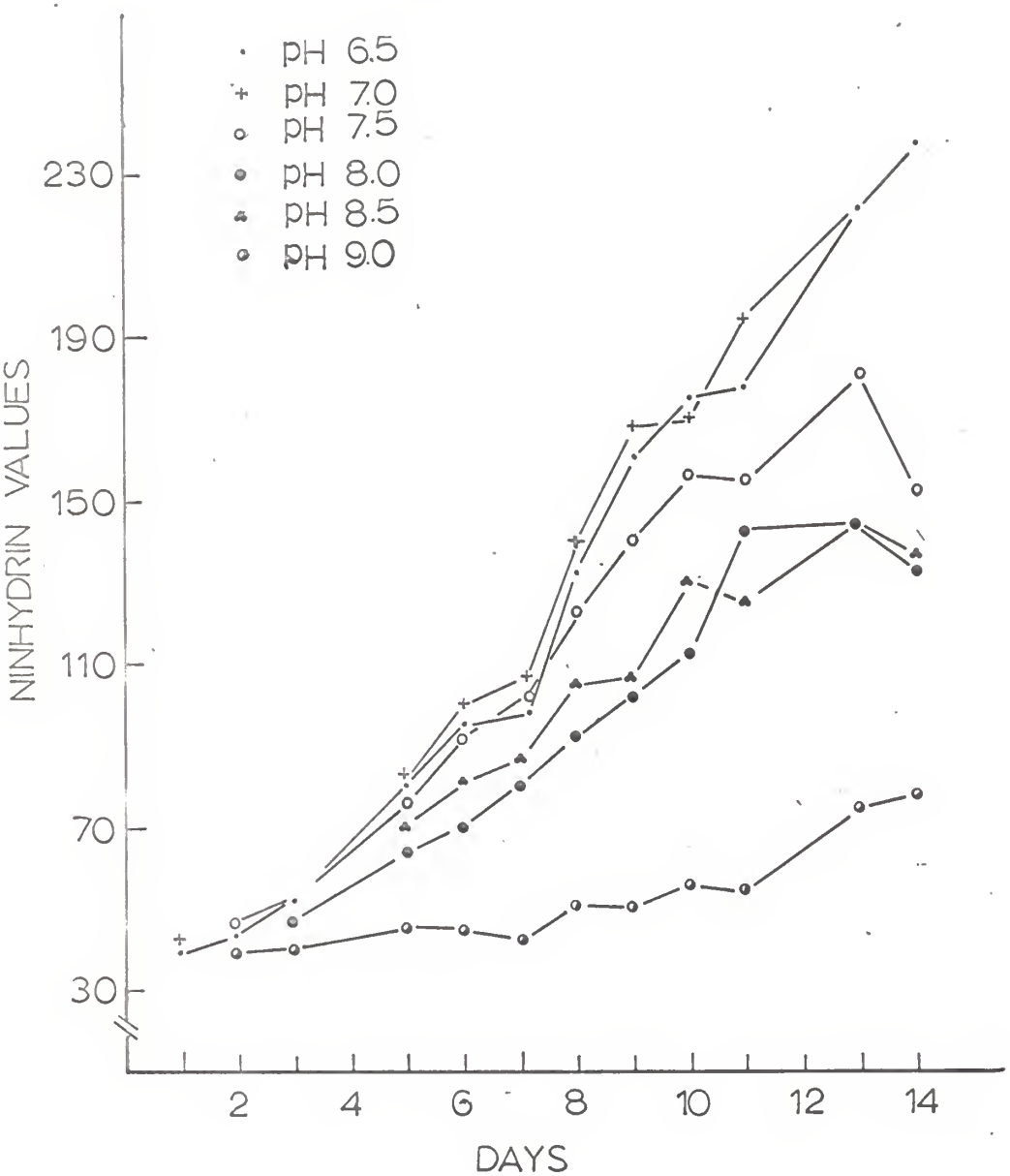


Fig. 2. Effect of pH on proteolysis of milk by Ps. fluorescens at 30 C. Bacterial growth occurred at all pH values.

pH was determined each day and at the end of 14 days incubation the pH values of all the flasks had become adjusted to within the range of 8.2 to 8.55.

Bacterial viability (on milk agar plates) did not appear to be affected by the high pH values as growth occurred throughout the experiment. The odor of the flasks with initial pH values above 7.0 was unclean and obnoxious. Thus, the adjustment of the milk medium to higher pH values was of no advantage since it did not create more rapid proteolysis or produce an acceptable odor.

Effect of Activators and Inhibitors

The flasks of inoculated 10% NFDM containing various additives (Co^{++}, Fe^{++}, Fe^{+++}, Mn^{++} and cysteine were incubated at 25 C for 5 days before being placed at 37 C. The addition of iron (as $FeSO_4 \cdot 7H_2O$ or $FeCl_3 \cdot 6H_2O$) gave increased proteolysis at all concentrations over the control flasks containing no iron except at 5 and 10 mg $FeCl_3 \cdot 6H_2O$/ml. This is shown in Table 1 and Table 2.

Table 1. Effect of $FeSO_4 \cdot 7H_2O$ on proteolysis of milk by Ps. fluorescens[a].

	Ninhydrin values[b] Mg $FeSO_4 \cdot 7H_2O$/ml of milk			
Days[c]	0	20	40	60
5	70	70	71	60
7	64	95	121	85
11	250	272	278	290

[a]Bacterial growth occurred at all levels.

[b]Absorbancy at 570 mu x dilution.

[c]Incubation at 37 C after 5 days initial growth at 25 C.

Bacterial growth (as shown on milk agar plates) ceased after 10 days in flasks containing $FeCl_3 \cdot 6H_2O$ but continued past the eleventh day in those containing $FeSO_4 \cdot 7H_2O$. The proteolyzed milk (with Fe) became dark brown within 3 to 5 days and its odor was that of spoiled cabbage.

Table 2. Effect of $FeCl_3 \cdot 6H_2O$ on proteolysis of milk by Ps. fluorescens[a].

	Ninhydrin values[b]			
	Mg $FeCl_3 \cdot 6H_2O$/ml milk			
Days[c]	0	1	5	10
4	89	105	104	89
6	140	141	151	141
8	213	236	214	210
10	295	312	295	290

[a] Bacterial growth ceased at 10th day of incubation at all levels.

[b] Absorbancy at 570 mu x dilution.

[c] Incubation at 37 C after 5 days initial growth at 25 C.

The data in Table 3 indicate that the compounds of $MnSO_4 \cdot H_2O$, cysteine and $CoSO_4 \cdot 5H_2O$ were apparently inhibitory to proteolysis. The control flask had higher ninhydrin values than those with added compounds. Bacterial growth on milk agar plates occurred at all levels except 0.1 M $CoSO_4 \cdot 5H_2O$ and growth was never initiated. The odor of the flasks was not unpleasant except for those containing cysteine and they had a skunk-like or rotten egg odor.

Of the compounds tried, only iron at a low concentration stimulated the proteolysis of 10% NFDM to any appreciable extent. However, the odor of the final product was very undesirable and hence, iron would appear to be an unsuitable stimulant from the standpoint of the ultimate objective of the research.

Table 3. Effect of $MnSO_4 \cdot H_2O$, $CoSO_4 \cdot 5H_2O$ and cysteine on proteolysis of milk by Ps. fluorescens.[a,b]

Compound	Days	Ninhydrin values[c] Mole of compound/ml of milk 0	0.1	0.01	0.001
$MnSO_4 \cdot H_2O$	5	86	32	52	69
	7	113	34	66	104
	9	156	37	88	126
	11	182	36	143	181
Cysteine	5	86	70	62	73
	7	113	94	88	112
	9	156	90	112	139
	11	182	90	136	178
$CoSO_4 \cdot 5H_2O$	5	86	31	28	35
	7	113	36	31	42
	9	156	37	32	54
	11	182	36	28	66

[a]Bacterial growth occurred at all levels except 0.1 M $CoSO_4 \cdot 5H_2O$ where growth was never initiated.

[b]Incubation at 25 C.

[c]Absorbancy at 570 mu x dilution.

Somevariations and apparent discrepancies in the ninhydrin values in the temperature, pH and activator studies may be due to the fact that the test measures alpha amino acids. Different enzymes may give different types of hydrolysis that might not be measured by the ninhydrin test. Also, there could be a browning reaction between amino acids and lactose which would bind the amino acids and cause a reduction in ninhydrin values.

Effect of Aeration

From earlier work, the growth of Ps. fluorescens was found to be greatest in a growth medium which was in thin, quiescent layers. Since the

organism is an obligate aerobe, different aeration techniques were tested. These included: bubbling air through the medium, releasing oxygen over the surface of the medium and agitation of the medium. All of the techniques proved unsuccessful as precipitation of the milk medium occurred and little proteolysis was evident.

Stage of Maximum Enzyme Elaboration

Plating methods were used in following cell proliferation and the proteolyzed zone technique was used as a screening method for proteolytic activity. These were not determined from the same flask, however. The highest viable cell count in flasks of 10% NFDM incubated at 25 C was from 4 to 7 days and at 14 days as can be seen in Figure 3. The number of viable cells increased rapidly and maintained a rather constant plateau after the first day of incubation until the end of the test. The zone diameters of proteolysis, however, were greatest at 4 to 8 days (Figure 4) and again at 13 days when the milk agar plates were incubated at 25 and 45C. The zones were greatest at the higher temperature of 45 C. The drop in the curve at the 9th day of incubation was probably due to an accidental addition of 1 ml of toluene to the incubating flask at that time. From these data, it was decided to grow Ps. fluorescens in 10% NFDM for 7 days at 25 C before beginning fractionation procedures on the proteolyzed growth medium for enzyme separation. It appeared this would be optimum for enzyme concentration.

Enzyme Concentration

The various treatments for attempted enzyme separation as described under Experimental Procedures resulted in the following five fractions:

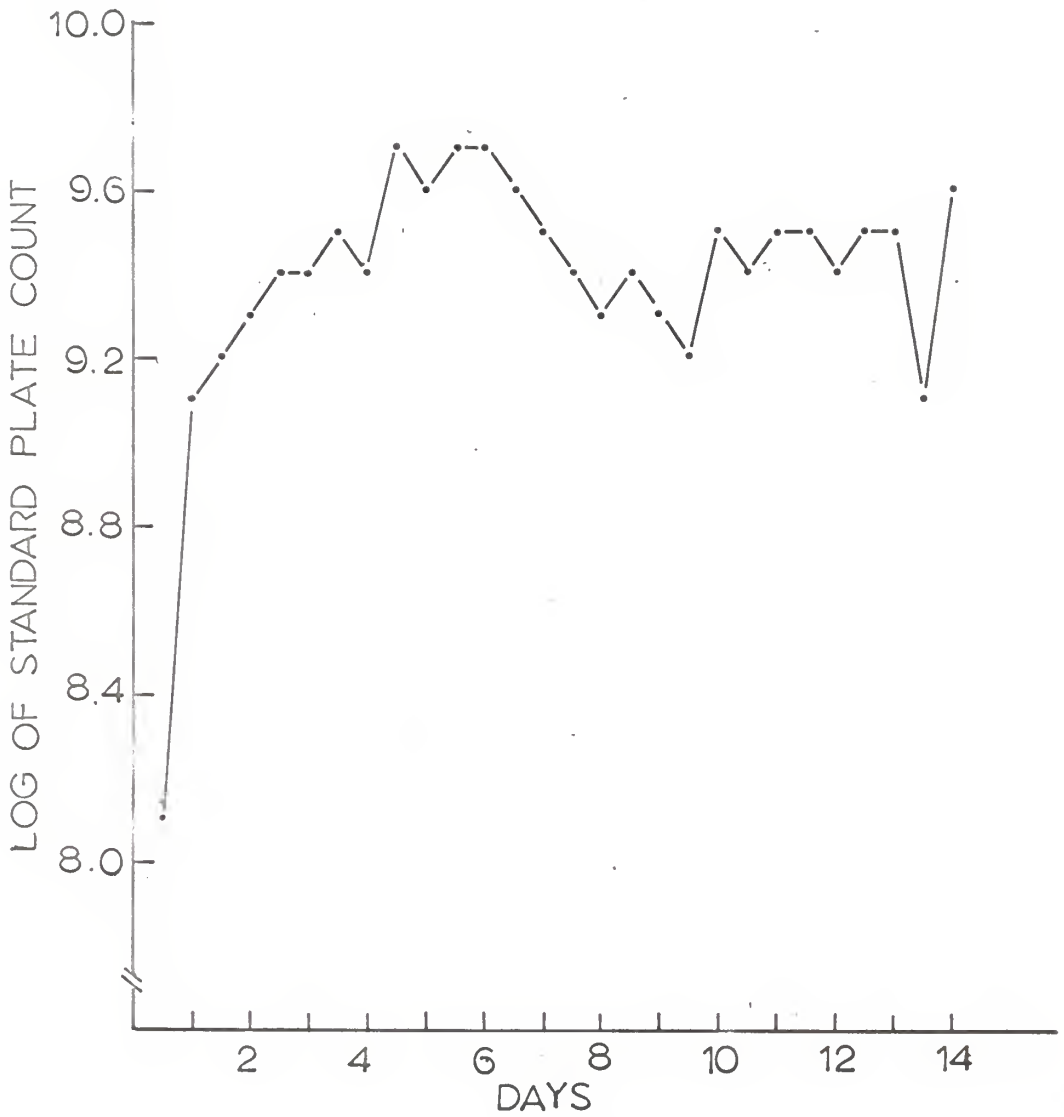


Fig. 3. Growth curve of Ps. fluorescens in 10% reconstituted NFDM. at 25 C.

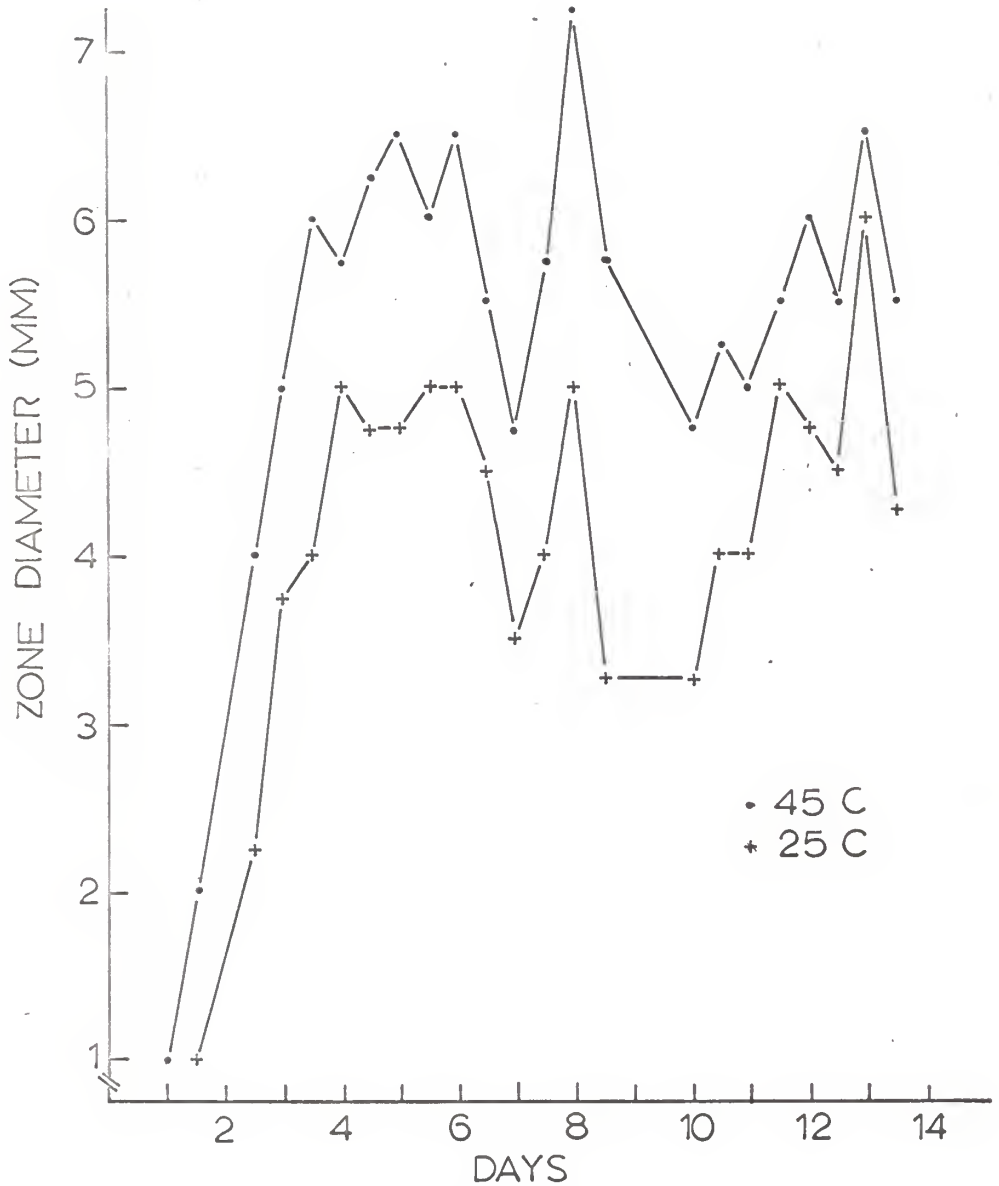


Fig. 4. Effect of incubation period on the proteolytic activity of growth medium inoculated with Ps. fluorescens. Activity was measured by diameter of proteolyzed zones on milk agar plates.

I, Untreated proteolyzed growth medium; II, Supernatant from centrifuged proteolysate; III, $(NH_4)_2SO_4$ precipitate dialyzed against pH 7.5 phosphate buffer (Na_2HPO_4, KH_2PO_4); IV, $(NH_4)_2SO_4$precipitate dialyzed against pH 7.0 phosphate buffer (Na_2HPO_4, NaH_2PO_4); and V, $(NH_4)SO_4$ precipitate dialyzed against pH 7.9 phosphate buffer (Na_2HPO_4, KH_2PO_4). A summary of the treatments used to achieve partial concentration of the proteolytic enzyme system is given in Tables 4 and 5 along with the results.

From the data in the tables, it can be seen that the proteolytic activities shown by the different fractions were not the same in each trial. A number of factors could be the cause of the variations. Fractionation procedures and testing methods are most likely. Activity (as specific activity) was lost after centrifugation.

Table 4. Proteolytic activity of partially purified enzyme fractions of Ps. fluorescens.

Trial A

Fraction	Volume	Mg N/ml	Mg tyrosine/ml	Specific[a] activity	Total activity	% Yield
I	930	5.50	0.48	0.087	446.4	100
II	760	5.28	0.28	0.053	212.8	41
III	200	5.85	1.235	0.211	247.0	55

[a]Mg tyrosine/ml ÷ mg N/ml.

The decrease in the mg N/ml of this fraction can be explained since some protein nitrogen was undoubtedly discarded in the precipitate. The mg tyrosine/ml (as determined from the enzyme activity test) also showed a decrease in activity. Perhaps a portion of the enzymes was precipitated during centrifugation and was lost when the precipitate was discarded. Also, the results from the enzyme activity test were measured in tyrosine units. Tyrosine may not be always the best measure of proteolysis.

Table 5. Proteolytic activity of partially purified enzyme fractions of Ps. fluorescens.

Trial B						
Fraction	Volume	Mg N/ml	Mg tyrosine/ml	Specific[a] activity	Total activity	% Yield
I	930	5.64	0.7	0.124	651.0	100
II	760	5.31	0.2	0.037	152.0	23
IV	71	6.70	1.13	0.167	80.2	12
V	65	5.64	1.05	0.186	68.3	10

[a]Mg tyrosine/ml ÷ mg N/ml.

The increase in specific activity of the dialyzed fractions over the untreated growth medium was not great. Perhaps the fractionation procedures were not the most suitable for separating the enzymes from a complex proteolyzed milk system medium. Many research workers use a less complex medium such as broth. The use of 1% NFDM as substrate in the enzyme activity test may have been a reason for the low activity results. Usually, hemoglobin or casein are used as substrates. However, since NFDM served as the growth medium in this work, it was thought that its use as the substrate in the enzyme activity test would give a more suitable measure of the activity of the enzymes in milk.

Other Fractionation Procedures

Various other procedures were used in attempts to obtain more active enzyme fractions. They included: preprecipitation by acid and heat, different centrifugation speeds, varying the $(NH_4)_2SO_4$ concentrations, solvent precipitation, disruption of bacterial cells with French Pressure Cell and column chromatography using Sephadex G-25. However, none gave any better results than fractionation with 50% $(NH_4)_2SO_4$.

Investigation of fractionation methods, critical evaluation of testing

methods and the use of different media for the growth medium and for substrates in the activity test should be considered in any future work.

SUMMARY AND CONCLUSIONS

The investigation was directed toward the acceleration of milk proteolysis by Ps. fluorescens and attempts to separate and concentrate the enzyme system. It was part of an overall project aimed at producing a beef-extract substitute.

In studying the effect of temperature, reconstituted NFDM inoculated with Ps. fluorescens was given an initial incubation at 25 C for 5 days. The purpose was to obtain bacterial proliferation and enzyme elaboration. Following initial incubation, proteolysis was most rapid at 40 C with 45 C closely approaching it. It was slowest at 25 C with somewhat increased proteolysis at 30 and 37 C. Bacterial growth at 45 C ceased after 3 days and after 4 days at 40 C. The proteolysates at 40 and 45 C had an undesirable odor which offset the advantage of more rapid proteolysis.

In trials on effect of pH at 30 C, proteolysis was most rapid at initial pH values of 6.5 and 7.0 and decreased with initial pH values of 7.5, 8.0, 8.5 and 9.0. Bacterial growth occurred throughout the experiment. The odor of the proteolysates was obnoxious and thus, adjustment of pH was of no advantage.

Investigations with activators showed that all concentrations of iron (except 5 and 10 mg $FeCl_3 \cdot 6H_2O$/ml) activated proteolysis. Bacterial growth occurred throughout most of the trials. The odor of the proteolysate containing iron was that of spoiled cabbage, however. The various types of aeration treatments retarded rather than accelerated proteolysis.

During fermentation, bacterial counts were highest at 4 to 7 days and

the proteolytic activity (as shown by proteolysis on milk agar plates) was greatest at 4 to 8 days and again at 13 days.

In attempts to cnncentrate the enzyme, a 7-day old proteolysate incubated at 25 C was used. The procedures included centrifugation, precipitation with 50% $(NH_4)_2SO_4$ followed by dialysis of the precipitate against phosphate buffer. These steps were conducted at 4 C or below. Specific activity of the various fractions was measured by the mg tyrosine released in a given time from a milk solids substrate per mg protein nitrogen in the enzyme fraction.

Although an increase in specific activity was obtained in the dialyzed precipitate it was much less than expected. Presumably, fractionation procedures were inadequate. Tests with other separation procedures gave no better results. Possibly the methods are less suitable with the complex milk medium than with a broth medium. It also may be that the testing method using milk solids is less satisfactory than those using casein or hemoglobin.

In future work, different media for the growth medium and substrates in the activity test should be considered. In addition, fractionation procedures and testing methods should be further examined.

ACKNOWLEDGEMENT

The author wishes to express her sincere appreciation to Dr. T. J. Claydon, Department of Dairy and Poultry Science, for his helpful suggestions, advice and counsel during the entire period of research, and for his assistance and guidance in preparing this thesis.

Appreciation is also extended to Professor Ross Mickelsen for his advice during the latter part of the study.

Acknowledgement is also extended to those persons who generously gave their advice and encouragement during the entire period of study at Kansas State University.

LITERATURE CITED

(1) American Public Health Association, Inc. Standard Methods for Examination of Dairy Products. 11th Ed., Am. Public Health Assoc., New York, N. Y. 1960.

(2) Anson, M.L. 1938. Estimation of Pepsin, Trypsin, Papain, and Cathepson with Hemoglobin. J. Gen. Physiol., 22: 79.

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

(4) Baribo, L.E. and Foster, E.M. 1952. The Intracellular Proteinases of Certain Organisms from Cheese and Their Relationship to the Proteinases in Cheese. J. Dairy Sci., 35: 149.

(5) Brandsaeter, E. and Nelson, F.E. 1956. Proteolysis by Lactobacillus casei. I. Proteinase Activity. J. Bact., 72: 68.

(6) Brandsaeter, E. and Nelson, F.E. 1956. Proteolysis by Lactobacillus casei. II. Peptidase Activity. J. Bact., 72: 73.

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

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

(9) Cordon, T.C. and Schwartz, J.H. 1962. The Fungus Beauveria tenella. Science, 138: 1265.

(10) Cowgill, R.W. and Pardee, A.B. Experiments in Biochemical Research Techniques. John Wiley and Sons, Inc., New York, N. Y. 1957.

(11) Dixon, M. and Webb, E. Enzymes. Academic Press Inc., New York, N. Y.,1958.

(12) Erickson, D.S. and Evans, D.A. 1958. Development of Flavors in Sterile Milk by Pure Cultures of Bacteria at Low Temperatures. (Abstract). J. Dairy Sci., 41: 341.

(13) Erlanger, B.F. 1957. Ornithine Peptidase of Bacillus brevis. J. Biol. Chem., 224: 1073.

(14) Friedman, M.E., Nelson, W.O. and Wood, W. 1953. Proteolytic Enzymes of Bacillus linens. J. Dairy Sci., 36: 1124.

(15) Fukumoto, J., Yamamoto, T. and Kazuhiro, I. 1960. Purification and Properties of the Neutral Proteinase of Bacillus subtilis. Nippron Nogei-Kagaku Kaishi 32, 230-3 (1958). (Chem. Abst., 54: 4758, 1960).

(16) Garibaldi, J.A. and Bayne, H.G. 1960. The Effect of Iron on the Pseudomonas Spoilage of Experimentally Infected Shell Eggs. J. Poultry Sci., 39: 1517.

(17) Grimberg, M. 1965. New Types of Rennet Preparation. Dairy Sci. Abstrs. 27: #3734.

(18) Groninger, H.S. and Eklund, M.W. 1966. Characteristics of a Trichosporon Species Isolated from Dungeness Crab Meat. Applied Microbiol., 14: 110.

(19) Harrow, B. and Mazur, A. 1962. Textbook of Biochemistry. W.B. Saunders Company, Philadelphia, Pa.

(20) Hull, M.E. 1947. Studies on Milk Proteins. II. Colorimetric Determination of the Partial Hydrolysis of the Proteins in Milk. J. Dairy Sci., 30: 881.

(21) Hurley, W.C., Gardner, F.A. and Van Der Zant, C. 1963. Characteristics of a Proteolytic Enzyme System of Pseudomonas fluorescens. J. Food Sci., 28: 47.

(22) Hurley, W.C., Gardner, F. A. and Van Der Zant, C. 1963. Growth and Proteolytic Activity of Pseudomonas fluorescens in Egg and Egg Products. J. Food Sci., 28: 114.

(23) Husain, I. and McDonald, I.J. 1958. Characteristics of an Extracellular Proteinase from Micrococcus freddenreichii. Canadian J. Microbiol., 4: 237.

(24) Koburger, J.A. and Claydon, T.J. 1961. Identification of Substances in Milk Cultures of Pseudomonas fluorescens which Stimulate Lactic Starter Cultures. J. Dairy Sci., 44: 1811.

(25) Kraft, A.A. and Ayres, J.C. 1961. Production of Fluorescence on Packaged Chicken. Applied Microbiol., 8-9: 549.

(26) Levinson, H.S. and Sevag, M.G. 1954. Manganese and the Proteolytic Activity of Spore Extracts of Bacillus megaterium in Relation to Germination. J. Bact., 67: 615.

(27) 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.

(28) McDonald, I.J. 1964. Characteristics of Proteinases of Three Strains of Staphylococcus lactis Isolated from Cheddar Cheese. J. Dairy Res., 31: 147.

(29) Nashif, S.A. and Nelson, F.E. 1953. The Lipase of Pseudomonas fragi. I. Characterization of the Enzyme. J. Dairy Sci., 36: 459.

(30) Pavlasova, E. and Starka, J. 1958. Preparation of Proteases formed by Bacillus subtilis and Bacillus mycoides. Folia Microbiol., 4: 308.

(31) Peterson, A.C. and Gunderson, M.F. 1960. Some Characteristics of Proteolytic Enzymes from Pseudomonas fluorescens. Applied Microbiol., 8: 98.

(32) Raksky, S.E.S.E. 1958. Studies on the Bacteriology of Egyptian Soft Cheeses. I. Isolation and Identification of Proteolytic Microorganisms. Indian J. Dairy Sci., 11: 11.

(33) Sadoff, H.L., Halvorson, H.O. and Finn, R.K. 1956. Electrolysis as a Means of Aerating Submerged Cultures of Microorganisms. Applied Microbiol., 4: 164.

(34) Sandvik, O. and Fossum, K. Accumulation of Bacterial Proteinases During Storage of Milk Due to the Selection of Psychrophilic Bacteria. Meieriposten 52 (28) 639-45 (1963). (Dairy Sci. Absts., 25, #3519).

(35) Shahani, K.M. and Sommer, H.H. 1951. The Protein and Non-Protein Nitrogen Fractions in Milk. I. Methods of Analysis. J. Dairy Sci., 34: 1003.

(36) Umbreit, W.W., Burris, R.H. and Stauffer, J.F. Manometric Techniques. Burgess Publishing Company, Minneapolis, Minn. 1964.

(37) Vadehra, D.V. and Harmon, L.G. 1965. Isolation and Purification of Staphylococcal Lipase. (Abstract). J. Dairy Sci., 48: 782.

(38) Van Der Zant, C. 1957. Proteolytic Enzymes from Pseudomonas putrefaciens. I. Characteristics of an Extracellular Proteolytic Enzyme System. Food Res., 22: 151.

(39) Van Der Zant, C. and Moore, A.V. 1955. Influence of Certain Factors on Bacterial Counts and Proteolytic Activity of Several Psychrophilic Organisms. J. Dairy Sci., 38: 743.

(40) Van Der Zant, C. and Nelson, F.E. 1953. Proteolysis by Streptococcus lactis Grown in Milk with and without Controlled pH. J. Dairy Sci., 36: 1104.

(41) Van Der Zant, C. and Nelson, F.E. 1953. Characteristics of an Endocellular Proteolytic Enzyme System of Streptococcus lactis. J. Dairy Sci., 36: 1212.

(42) Van Der Zant, C. and Nelson, F.E. 1954. Characteristics of Some Endocellular Peptidases of Streptococcus lactis. J. Dairy Sci., 37: 795.

(43) Vorbeck, M.L. and Cone, J.F. 1963. Characteristics of an Intracellular Proteinase System of a Trichosporon Species Isolated from Trappist-Type Cheese. Applied Microbiol., 11: 23.

(44) Whitaker, J.R. 1957. Properties of the Proteolytic Enzymes of Commercial Ficin. J. Food Res., 22: 483.

(45) Williamson, W.J., Tove, S.B. and Speck, M.L. 1964. Extracellular Proteinase of Streptococcus lactis. J. Bact., 87: 49.

(46) Zittle, C.A. 1965. Purification of Protease in Cow's Milk. J. Dairy Sci., 48: 771.

SOME FACTORS AFFECTING PROTEOLYSIS OF MILK BY *PSEUDOMONAS FLUORESCENS*

by

ROSA LUELLA THOMAS

B. S., West Virginia University, 1964

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

1967

This investigation was part of an overall project designed to produce a beef-extract substitute. Its objectives were to accelerate proteolysis of milk by Pseudomonas fluorescens and to separate and concentrate the proteolytic enzyme system. Reconstituted nonfat dry milk (NFDM) was autoclaved in Erlenmeyer flasks, inoculated with Ps. fluorescens, and incubated for periods up to 14 days under various conditions. The extent of proteolysis was determined by measuring ninhydrin reactive compounds at 570 mu with a Spectronic 20.

In studying the effects of incubation temperature, the fermentation was carried out initially at 25 C for 5 days in order to obtain bacterial development and enzyme production. After this period, flasks were placed at 45, 40, 37, 30 and 25 C. Proteolysis was most rapid at 40 C followed closely by that at 45 C. At the remaining temperatures, proteolysis became progressively slower as the incubation temperature decreased. Bacterial growth (as shown by streakings on milk agar plates) ceased after 3 days at 45 C and after 4 days at 40 C. As incubation temperatures increased, the odor of the proteolysate became more objectionable.

The adjustment of inoculated milk at 30 C to initial pH values ranging from 6.5 to 9.0 resulted in a gradual decrease in the rate of proteolysis with increasing pH. Values of 6.5 and 7.0 gave most rapid proteolysis. Bacterial growth occurred at all pH values. The odors of the proteolysates at the high pH values were obnoxious. Of compounds tested for activation, iron was the only one that stimulated proteolysis (except at concentrations of 5 and 10 mg/ml of $FeCl_3.6H_2O$). Bacterial growth occurred throughout the trials

with the various additives except at 0.1 M $CoSO_4.5H_2O$. Any advantage obtained through accelerated proteolysis was offset by a disagreeable odor of spoiled cabbage.

Bacterial counts in flasks of inoculated NFDM incubated at 25 C were highest at 4 to 7 days. The proteolytic activity (as shown by proteolysis on milk agar plates) was greatest at 4 to 8 days and again at 13 days.

For enzyme concentration work, a 7-day old proteolysate incubated at 25 C was used. The following procedures were conducted at 4 C or below: centrifugation of the proteolysate, precipitation with 50% $(NH_4)_2SO_4$ and dialysis of the precipitate against phosphate buffer. Specific activity of the various fractions was measured by the mg tyrosine released in a given time in a milk solids substrate per mg protein nitrogen in the enzyme fraction.

The increase in specific activity obtained in the dialysed precipitate over the original proteolysate was slight. This may have been due to inadequate fractionation procedures and testing methods. Milk solids, used as the growth medium and as the substrate in the testing method were, perhaps, too complex for enzyme separation techniques and for rapid enzyme activity measurement. Several other separation procedures were tried but none gave any better results than 50% $(NH_4)_2SO_4$.

Critical evaluation of testing methods, investigation of fractionation methods and the use of different media for the growth medium and for substrates in the activity test should be considered in any future work.