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Title PURIFICATION AND KINETICS OF GELATINASE OBTAINED FROM AN OBLIGATELY PSYCHROPHILIC MARINE VIBRIO

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Optimum growth of Marine Psychrophile 41 (MP-41) an obligately psychrophilic marine vibrio, occurred at 18 C and pH 8.0 to pH 8.3.

Twenty eight-fold purification of gelatinase from this organism was effected by ammonium sulfate fractionation and column chromatography on Sephadex G-200 gel.

The effects of temperature, pH, pressure, and salt concentration were determined for various degrees of enzyme purity and for cells. Differences occurred in the various enzyme preparations with optimal purified gelatinase activity observed at 40 C and pH 9.3. Increasing pressures and salt concentrations decreased the activity of purified gelatinase but salt-free purified gelatinase displayed no activity. The rate of hydrolysis was greater at 40 C than at 5 C. At the lowest gelatinase and gelatin concentrations studied, 0.025 mg/ml and 1 mg/ml respectively, activity was observed although optimal

activity occurred at higher concentrations.

Ammonium sulfate was shown to increase the activity of purified gelatinase two-fold.

PURIFICATION AND KINETICS OF GELATINASE OBTAINED FROM AN OBLIGATELY PSYCHROPHILIC MARINE VIBRIO

by

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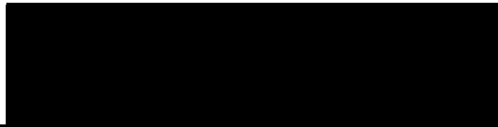

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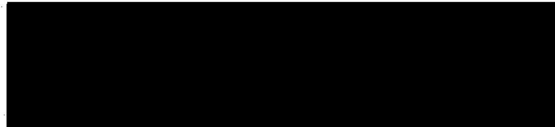

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PURIFICATION AND KINETICS OF GELATINASE OBTAINED FROM AN OBLIGATELY PSYCHROPHILIC MARINE VIBRIO

INTRODUCTION

Although the cycles of matter are known to operate in the ocean, very little research on the actual operational mechanisms of these cycles has been undertaken. Generally the parts attributed to the bacteria are axiomatically assumed. These assumptions are based upon the knowledge gained by soil microbiologists. Unfortunately, marine microbiologists have assumed that the elucidation of a microorganism in any given environment is sufficient to assign biochemical activities to it. However, in the deep sea the parameters of low temperature and increased hydrostatic pressure may affect the biochemical activity of microorganisms. The large volume of water below the thermocline as well as at both poles makes low temperature an important environmental factor that must be taken into consideration when dealing with the activities of microorganisms in the sea. Furthermore, in oceanic waters the concentration of bacteria is known to be low, and much of the biochemical action has been attributed to the action of free enzymes. The existence of free enzymes has not been demonstrated conclusively in the marine environment.

This investigation deals with one of the many steps in the

nitrogen cycle, proteolysis. This reaction must occur with proteinaceous material resulting from the death of micro- and macro-organisms. The selection of gelatinase as the proteolytic enzyme to study was made on the following bases: it is easily assayed; it is an exoenzyme which may exist as a free enzyme; it is produced by most marine bacteria including obligate psychrophiles; and the system lends itself to kinetic studies.

REVIEW OF LITERATURE

The bacterial count of the open sea is often as low as one or two cells per ml (44). Yet the changes necessary for release of inorganic nutrients in the cycles of various elements are attributed to bacteria (14, p. 60 - 79). Thus the question of whether the small number of detected organisms is capable of bringing about these vast transformations has evolved (47, 48).

Kreps (22), after passing sea water samples through Seitz filters or sterilizing the samples with mercuric chloride, observed changes in the nitrite and ammonia content. Similar results were obtained by other researchers (8, 20). The variation in phosphate content of stored sea water is reduced but not halted by saturation with chloroform (32). Bokova and his associates (2) found changes in the oxygen, phosphate, nitrate, and ammonia content of Seitz-filtered sea water.

These changes in sterilized sea water have led to the speculation that free enzymes of bacterial origin are responsible for inorganic nutrient release (20, 22). ZoBell (50) has suggested that microbiological processes in sediments are also the result of free enzyme accumulations.

As a group, marine bacteria are actively proteolytic, attacking most proteinaceous material (51).

Detection of bacterial proteolysis is commonly accomplished through analysis of degradation products resulting from the activity of cell suspensions or culture filtrates on protein preparations. Casein, crystalline insulin, hemoglobin, lactoglobulin, and gelatin have been utilized with marine bacteria (26, 37, 38). Decolorization of the chromoproteins of *Porphyra*, when digested by growing colonies and suspensions, filtrates, or enzyme preparations of marine organisms, has been demonstrated by Merkel and his associates (27, 28).

Investigations on culture filtrates and purified enzyme preparations of the proteolytic marine species, *Aeromonas proteolytica*, sp. n., isolated from the alimentary canal of *Limnoria*, have provided information on temperature, pH, and other parameters affecting activity and stability (29, 36, 37). Similar studies have been carried out on unpurified supernatant enzymes from psychrophilic organisms of Arctic sediments (26).

Gelatin liquefaction was observed in the majority of strains tested from sediment, water, and fish samples (3, 7, 49). ZoBell has stated that three-fourths of the marine bacteria liquefy gelatin (51).

The classic method of determining gelatin degradation is the gelatin stab, incubated at refrigeration temperatures, and observed for liquefaction. The necessity for low incubation temperatures

often results in a slow reaction and therefore the lack of quantitative data. Stone (41) suggested incubating an inoculated tube of gelatin at 37.5 C or the optimum growth temperature. After incubation, the tubes are cooled to determine if the gelatin is still capable of solidification. Evaporation of moisture or short incubation periods could obscure a slow reaction.

Direct detection which can be used at regular incubation temperatures was demonstrated by Smith and Goodner (38). With various gelatin-agar media, gelatinase activity turns turbid media clear while clear media becomes cloudy in a zone around the colony. Merkel (27) has criticized this method because of difficulty in detecting the zones of change.

Several methods have been described which involve zonal changes in agar-gelatin media after the addition of protein precipitates. A gelatin reaction is indicated by a clear zone when the plate is flooded with an acid solution of mercuric chloride (11), saturated ammonium sulfate (5), or sulfosalicylic acid (9, p. 152-153). Flooding the plate with tannic acid produces a clear zone if the colony has slight gelatinase activity while extensive activity results in a white precipitate (11). Chapman (6) incorporated ammonium sulfate into a gelatin agar media with clear areas developing in the opaque medium around active colonies.

The viscosity of a gelatin medium drops with liquefaction.

The method of Levine and Carpenter (25), measuring this viscosity change, has proven too complex for routine laboratory use.

These investigators (25) also measured the increase in ammonia and amino acids caused by gelatin liquefaction with a formol titration.

The slide microtest of Thirst (42) detects only readily formed gelatinase present in the inoculum, but small quantities of culture grown under any conditions can be used. When microscope slides covered with gelatin are stained with carbol fuschin, gaps appear where gelatinase-producing organisms have been spotted.

A heat stable, formalin-denatured charcoal-gelatin strip placed in a test tube with media will dissolve under the influence of gelatinase leaving a black charcoal sediment on the bottom of the tube (21). The strip will also dissolve if placed on the surface of solid media. Lautrop (23) suggested placing the gelatin strip in a heavy suspension of established cultures to further speed the reaction.

The disappearance of the black layer from a strip of photographic paper placed in the culture media will also demonstrate gelatin proteolysis (24).

Gelatinase, like all proteins and enzymes, is subject to denaturation with rising temperatures. This denaturation results in a volume increase (ΔV) which is retarded or reversed by hydrostatic pressures. Considering a column of water ten meters deep to be

equivalent to approximately 1 atm pressure, about 84 percent of the ocean bottom is under pressures of 200 atm or greater (4). Thus pressure-temperature relationships and their effects on protein volume change and enzyme activity are of importance in the marine environment. The classic work of Johnson, Eyring and Polissar (18) discusses temperature and pressure in relation to protein (enzyme) denaturation and activity. Webb (46) also provides extensive background on the subject, while Johnson (17) and Hédén (15) relate temperature-pressure effects to the microbial environment.

MATERIALS AND METHODS

The Organism

Marine psychrophile 41 (MP-41), a Gram negative vibrio isolated from waters off the Oregon coast, was used in this work. Additional information on the organism, prepared by Colwell, is located in the Appendix. A stock culture was maintained in SDB broth, pH 7.8, at 5 C and transferred twice a month.

SDB broth consisted of 15 g NaCl, 5.0 g Rila Marine salts (Rila Products, Teaneck, N. J.), 0.5 g glucose, 0.2 g succinic acid, 3.0 g yeast extract (Difco), 0.01 g ferrous sulfate, and 5.0 g polypeptone (BBL) dissolved in one liter distilled water. After adjustment of the pH to pH 7.8, unless otherwise indicated, with NaOH, sterilization was effected by autoclaving for 20 minutes at 15 psi. All media were sterilized in this manner.

Growth Studies

To determine the optimum temperature of growth for this organism, a one percent inoculum of a 24 hour MP-41 culture grown in SDB broth, pH 7.25, at 15 C was inoculated into tubes containing SDB broth and incubated at various temperatures in a polythermostat. Growth was estimated by observing the change in optical density (OD)

at 600 mμ on a Bausch and Lomb Spectronic 20 colorimeter.

The same medium and procedure using various pH's, were employed to determine the optimum pH for growth. The temperature of incubation was 15 C.

Throughout this investigation all media as well as substrate, buffers, etc. were equilibrated to the appropriate temperatures befor use.

MP-41 was incubated at 15 C to obtain cell yields because of the inavailability of laboratory incubators which could be set at the optimum growth temperature.

Enzyme Preparation

Gelatinase was produced by growing the organism on nutrient gelatin (4. 0 g nutrient broth, 100 g gelatin (Difco), 21. 5 g NaCl, 5. 0 g Rila Marine salts, and one liter distilled water, pH 8. 0).

A thin layer (125 ml) of nutrient gelatin just covering the bottom of a culture flask (2500 ml) was inoculated with 50 ml of a 24 hour MP-41 culture growing in SDB broth, pH 7. 8, at 15 C. The 24 hour culture was prepared by inoculation of 100 ml broth with 1. 0 ml MP-41 stock culture. The culture flask was then swirled to distribute the inoculum over the entire surface of the nutrient gelatin. Incubation at 15 C followed. At approximately 36 hour intervals 100 ml sterile Rila buffer were added to the flask to prevent toxic

concentrations of metabolic by-products. Incubation continued until the entire system was liquefied or nearly so (four to seven days following inoculation).

Following incubation, the entire contents of the culture flask were centrifuged in a Sorvall RC-2 refrigerated centrifuge at 8,000 x g for 30 minutes to remove cellular matter and solid portions of the media. The supernatant, used as a gross enzyme preparation in activity assay or a step in the preparation of purified enzyme, will be referred to as supernatant gelatinase.

A preparation of Rila Marine salts (26.5 g Rila Marine salts per liter distilled water, self buffering at pH 7.9) was used for washing cells, as eluent in chromatographic procedures, and in controls during enzyme assays. Hereafter this preparation will be referred to as Rila buffer.

The supernatant gelatinase was fractionated with ammonium sulfate (12) and the active precipitates occurring between 35 - 50 percent saturation were recovered by centrifugation at 8,000 x g for 30 minutes, pooled, and stored. This precipitate was dissolved in Rila buffer and used for further enzyme determinations or as another step in enzyme purification. It will be referred to as fractionated gelatinase.

Column chromatography of an appropriate amount of fractionated gelatinase dissolved in a minimal amount of Rila buffer was

performed on Sephadex G-200 gel in Rila buffer (35). A column with a Buchler polystaltic pump supplied a continuous flow of eluent (0. 5 ml per minute) through the column of Sephadex. The gel was placed in buffer at least three days before packing the Pharmacia column (2. 5 cm diameter by 45 cm) to a height of about 8 cm. Buffer passed through the system for at least 24 hours prior to sample application to allow settling of the gel. The eluent was continuously monitored at 254 mμ by an ISCO Model UA recording ultraviolet analyzer. Fractions were collected manually by observing the absorbance pattern. The fraction between the first rise in absorbance until the start of the decline in the first peak was saved as the active fraction. The void volume of the column was determined using Blue Dextran 2000 (Pharmacia Fine Chemical Company).

Following chromatography the active fraction was brought to 50 percent ammonium sulfate saturation and stored until the contents of eight fermentation flasks had been prepared. These active portions were then pooled and stored until required. For use, about 3 mg of the enzyme (10. 0 ml of the pooled precipitated purified gelatinase evenly dispersed in the ammonium sulfate saturated Rila buffer) was removed, centrifuged at 17, 000 x g, decanted to remove the ammonium sulfate saturated buffer, and resuspended in Rila buffer. This partially purified enzyme will be referred to as purified gelatinase.

Fractionated and purified gelatinase preparations were stored in Rila buffer containing 313 g ammonium sulfate per liter (50 percent saturation). All enzyme preparation and storage procedures were carried out at 15 C except chromatography which was done at room temperature.

Salt-free gelatinase was prepared by dialysis of 50 percent ammonium sulfate saturated purified gelatinase against distilled water until neither the contents of the dialysis bag nor the surrounding water gave a positive result on direct Nesslerization. This enzyme is referred to as dialyzed gelatinase.

Methods of Gelatinase Activity Assay

These studies utilized the enzymatic hydrolysis of gelatin by the gelatinase to release free amino acids which were analyzed by ninhydrin assay, a modification of that described by Spies (39) and Moore and Stein (30). Due to the high glycine composition of gelatin (1) the ninhydrin was standardized to glycine (0.15 mg to 1.50 mg per ml concentrations), and a standard curve prepared. Protein hydrolysis was expressed as the amount of split products formed in terms of the quantity of glycine giving the same color reaction. The unit of enzyme activity as defined for this work was mg of glycine produced per mg gelatinase per hour.

Gelatin substrate for enzyme analysis was a 0.5 percent

gelatin medium consisting of 5. 0 g gelatin, 21. 5 g NaCl, 5. 0 g Rila marine salts and one liter distilled water at pH 7. 8 or pH 8. 0.

Five ml of the substrate was equilibrated in a test tube at the proper assay temperature and then 1. 0 ml of the enzyme preparation was added. After incubation for the appropriate time interval, 1. 0 ml ninhydrin solution was added and the tube placed in a steamer for 20 minutes. Two ml diluent (equal volumes of distilled water and reagent grade n-propanol mixed) were used to dilute the mixture upon removal from the steamer. Color development took 15 minutes and the absorbance was determined at 570 mμ within an hour. The original assays with supernatant and fractionated gelatinase were read on the Bausch and Lomb Spectronic 20 colorimeter while assays with purified gelatinase employed the Beckman Model DU spectrophotometer for absorbance determination.

Enzyme (5. 0 ml Rila buffer and 1. 0 ml enzyme) and gelatin (5. 0 ml gelatin and 1. 0 ml Rila buffer) controls as well as a zero time reading were run with each assay. Where necessary the assay systems were diluted with diluent to facilitate obtaining spectrophotometer readings and calculations were appropriately adjusted.

The ninhydrin solution was prepared by dissolving 4. 0 g ninhydrin (1, 2, 3-triketohydrindene, Eastman Organic Chemicals) in 500 ml 0. 2 M citrate buffer (21. 00 g citric acid monohydrate dissolved in 200 ml N NaOH diluted to 500 ml with distilled water,

pH 5.0). This was added to 500 ml citrate buffer in which 0.80 g reagent grade $SnCl_2$-$2H_2O$ had been dissolved. The ninhydrin solution was then decanted after settling to remove a white precipitate which formed upon mixture of the two citrate buffer solutions. Since the ninhydrin solution is unstable after preparation it was freshly prepared and standardized every two weeks and stored at 15 C.

A Rila buffer suspension of approximately 1.0 OD in a 1.0 cm cuvette prepared from a 24 hour culture of MP-41 cells grown in nutrient gelatin at 15 C and twice washed with Rila buffer was utilized for determination of MP-41 gelatinase activity. One ml of the cell suspension and 5.0 ml substrate (nutrient gelatin substrate) were incubated under appropriate conditions and then assayed by the ninhydrin method. Prior to reading absorbance, but following addition of diluent, the systems were filtered through Whatman Number 2 filter paper to remove cell debris.

Protein Determination

Protein estimations were by the method of Warburg and Christian (45).

Temperature Studies

A polythermostat constructed by Morita and Haight (31) and similar to that of Oppenheimer and Drost-Hansen (33) was used.

The temperature ranges were adjusted between 6 C and 51 C depending upon the particular experimental requirements. Thermally equilibrated test tubes containing media or substrate were inoculated with the proper cell or gelatinase preparation. After the experimental incubation interval the assay for the particular experiment was performed.

Pressure Studies

ZoBell and Oppenheimer (53) have described the apparatus used for incubation under hydrostatic pressure. Prior to use the cylinder was filled with water and equilibrated at experimental temperature. One ml gelatinase or cell preparation and 3 ml substrate were added to 10 mm by 38 mm test tubes, stoppered to exclude air bubbles, and placed in the pressure cylinder. The cylinder cap was secured and the pressure increased to the desired level. After the incubation period, the pressure was released and 2. 0 ml Rila buffer added to each reaction mixture. A ninhydrin assay was then performed. Although approximately ten minutes were required to complete the pressurization technique for all cylinders in a particular experiment, the release of pressure and assay were timed to provide the proper incubation interval.

Miscellaneous Gelatinase Assays

Studies involving substrate salt concentrations, pH, and gelatin content were carried out by altering the substrate as necessary while in the enzyme concentration activity assays the amount of gelatinase used was altered.

RESULTS

Cell Studies

Spot inoculation of gelatin medium with all of the stock cultures of psychrophiles in the laboratory and subsequent determination of gelatinase resulted in the selection of MP-41 as the organism for this study. The zone of liquefaction was one of the largest exhibited by the psychrophiles indicating the most extensive gelatinase production, activity, or both.

Since very little work had previously been done with MP-41, the optimal temperature and pH for growth were first determined. The optimal growth temperature for MP-41 was shown to be about 18 C with no growth above 25 C (Figure 1). Additional growth temperature studies showed growth to occur slowly at 0 C.

A pH of between pH 8.0 and pH 8.3 proved optimal for growth of the organism (Figure 2). Six hour studies showed growth to be slightly better at pH 8.3 while at 12 hours the pH 8.0 to pH 8.3 range exhibited approximately the same amount of growth. Further incubation produced no change in the pattern of growth for the 12 hour curve.

Ninhydrin assay of MP-41 gelatinase activity of washed cells at varying temperature and pH values was determined. A temperature range of 5 C to 24 C was used. Increased temperatures resulted

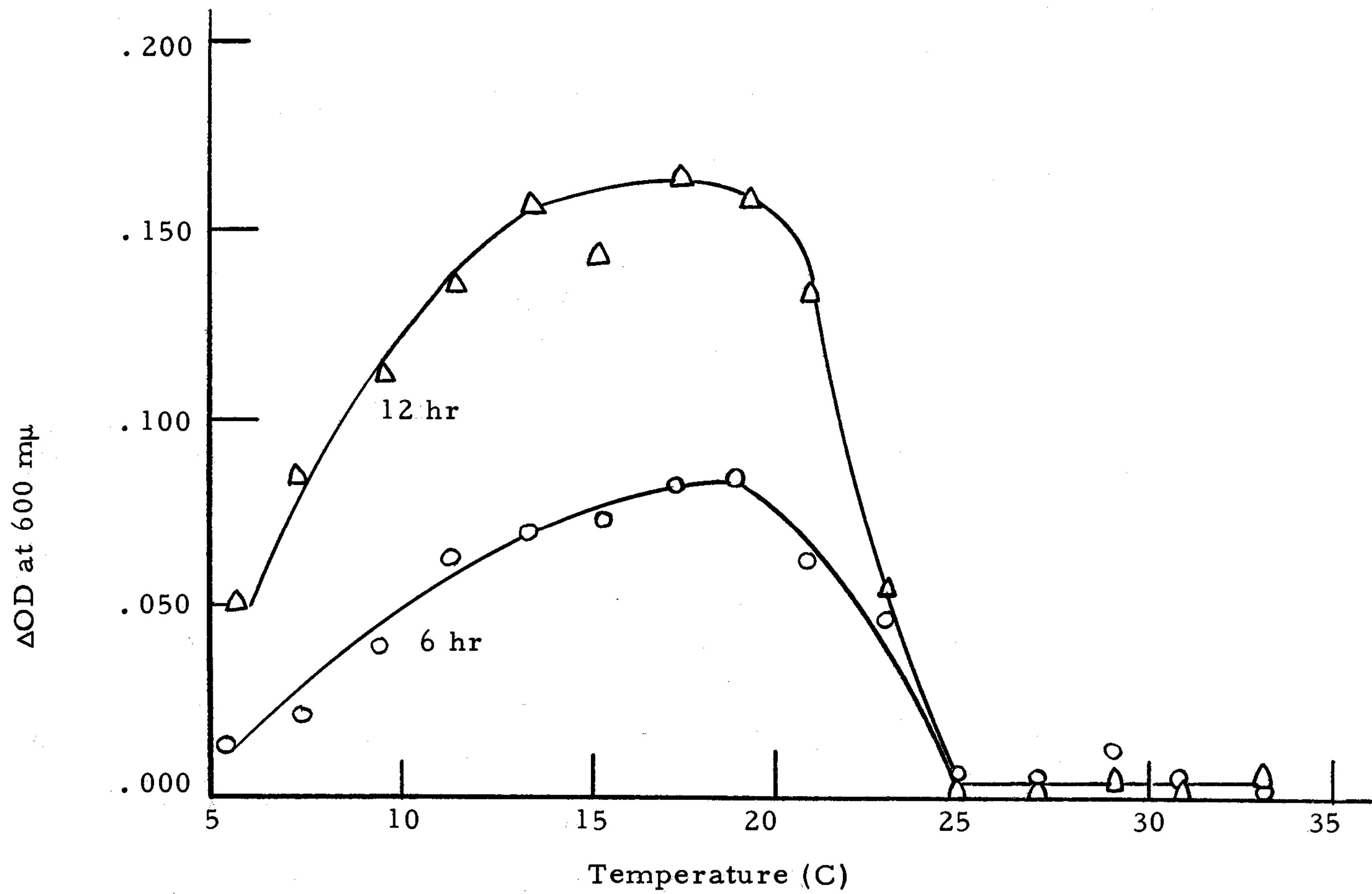

Figure 1. The effect of temperature on growth of MP-41. A 1 percent inoculum of a 24 hour culture of MP-41 grown at 15 C was introduced into SDB broth, pH 7.25. Growth was estimated by observing optical density change at 600 mμ with a Bausch and Lomb Spectronic 20 colorimeter. All tubes were equilibrated to the proper temperature before incubation. All values are the average of 4 tubes.

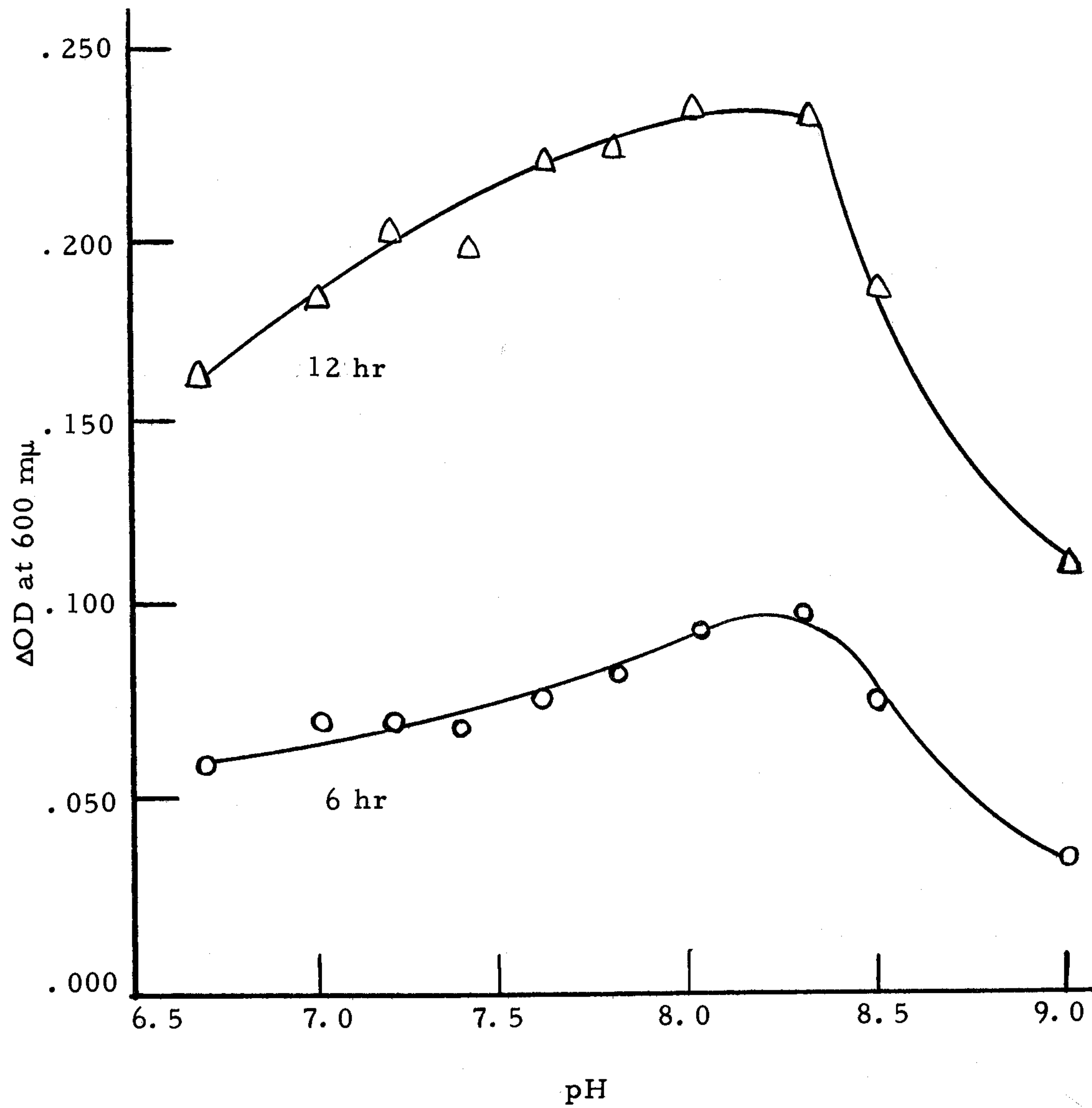

Figure 2. The effect of pH on MP-41 growth. Incubation was at 15 C in SDB broth at indicated pH values. Inoculum and growth estimation are the same as Figure 1. All values are the average of two tubes.

in greater gelatinase activity. At the highest temperature used in this experiment, which was also the maximum for cell growth (ca. 24 C), gelatinase activity was observed to be maximal (Figure 3). Nutrient gelatin substrate ranging in pH from 7. 0 to 9. 5 was employed for similar studies on the effects of pH. Examination of Figure 4 shows the optimum pH to be 8. 8 with no activity occurring at pH 7. 6 or lower.

Cells incubated at pressures ranging from 1 to 600 atm and 10 C showed little change in activity between 1 and 200 atm (Figure 5). Between 200 and 300 atm there is a drop in the activity. Thereafter the activity remained constant to 600 atm. At 15 C a slight activity drop occurred at 100 atm and activity was then fairly constant up to 600 atm. At 1 atm and 15 C, 0. 7 mg glycine more per hour was produced enzymatically than at 10 C. At 600 atm the activity difference is about 0. 5 mg glycine per hour illustrating a greater overall drop in activity at 15 C than at 10 C between 1 and 600 atm pressure. A control for the time necessary for pressurization showed no significant activity.

Ammonium Sulfate Fractionated Gelatinase Activity

Ammonium sulfate fractionation was carried out on the supernatant gelatinase. Table 1 shows the preliminary results of successively narrower fractionation. By bracketing the ammonium sulfate

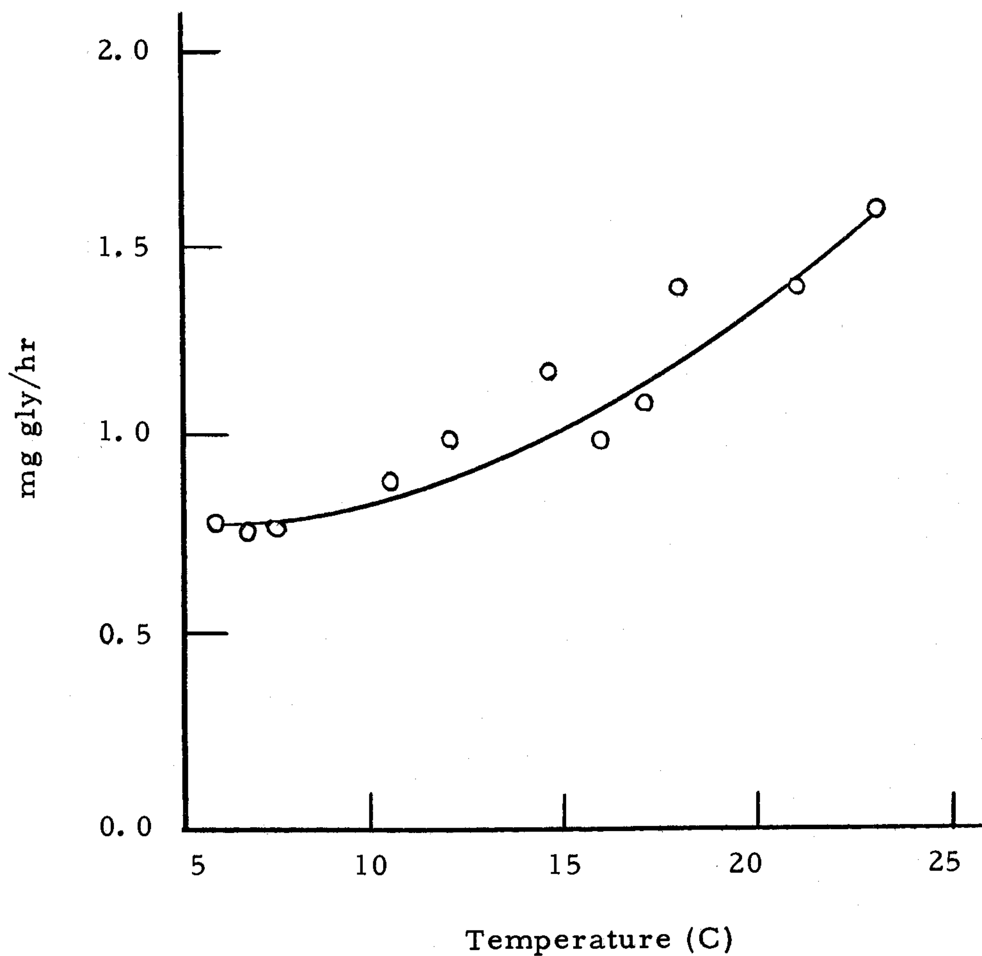

Figure 3. The effect of temperature on gelatinase activity of MP-41 cells. The reaction mixture contained 1 ml of a Rila buffer suspension (OD about 1.0 in a 1.0 cm cuvette) of twice-washed (Rila buffer) MP-41 cells grown in nutrient gelatin, pH 8.0, for 24 hours and 5 ml nutrient gelatin (0.5 percent) substrate, pH 8.0. Incubation was 4 hours in the polythermostat. The values are the average of 3 tubes.

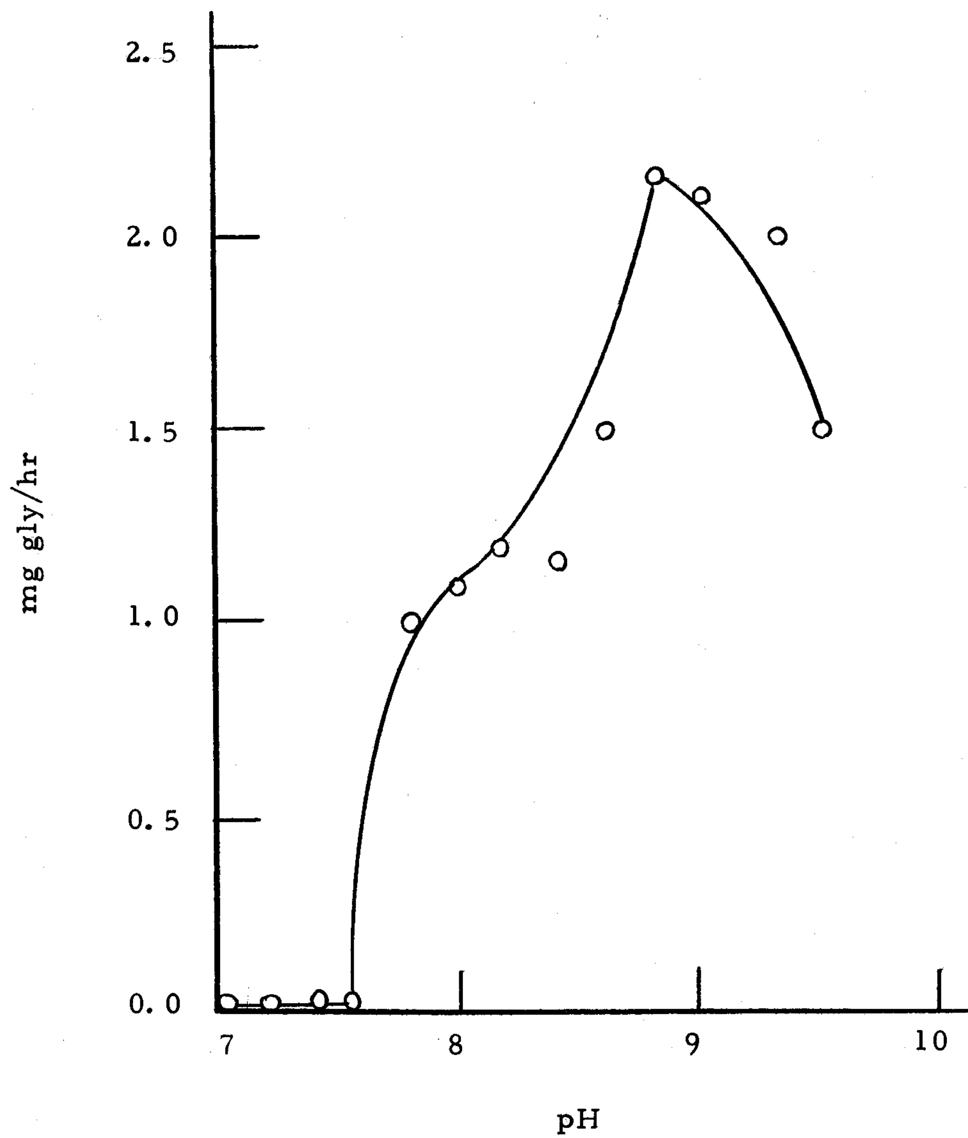

Figure 4. The effect of pH on gelatinase activity of MP-41 cells. The reaction mixture was as in Figure 3 except that the nutrient gelatin substrate was at the indicated pH values. Incubation was at 15 C for 4 hours.

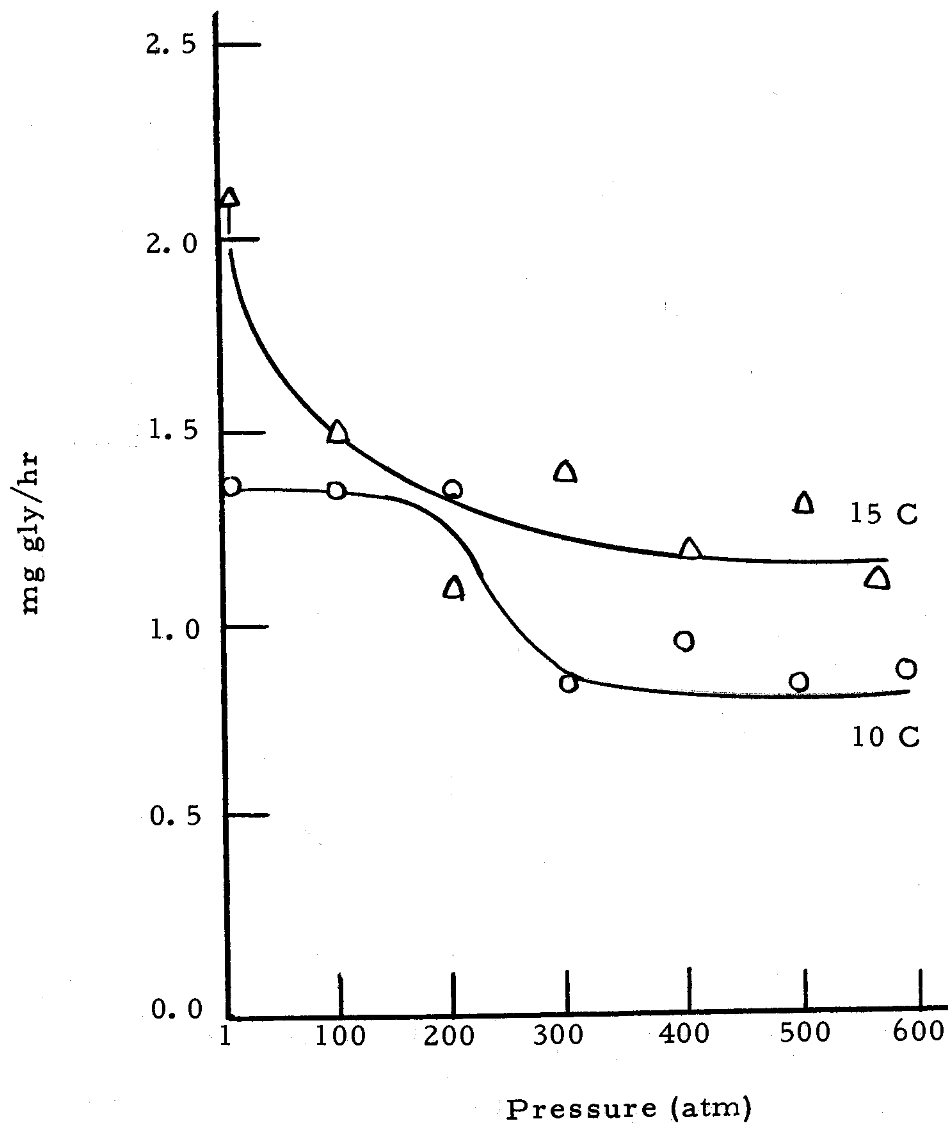

Figure 5. Pressure effects on gelatinase activity of MP-41 cells at 10 C and 15 C. The reaction mixture contained 1 ml cell suspension (as in Figure 3) and 3 ml nutrient gelatin substrate, pH 8. 0, and was brought to a 6 ml volume after incubation with 2 ml Rila buffer. Incubation was performed in 10 mm by 38 mm test tubes within pressure cylinders for 2 hours at indicated pressures and 10 C or 15 C.

fractionation process, it was decided to use the 35 - 50 percent fraction of supernatant gelatinase containing about 82 percent of the enzyme (activity) present in the supernatant gelatinase.

Table 1. Activity of fractions of supernatant gelatinase*

Fraction (%)	Activity (%)
0 - 40	77
40 - 60	19
60 - 80	3
80 - 100	1
0 - 30	5
30 - 40	29
40 - 50	61
50 - 60	5
30 - 35	4
35 - 40	46
40 - 45	24
45 - 50	26

*The reaction mixture contained 1 ml of the ammonium sulfate fraction precipitates dissolved in a minimal amount of Rila buffer and 5 ml gelatin (5 mg/ml) substrate, pH 7. 8. Incubation was 24 hours at 15 C.

No activity occurred in any gelatinase originally dissolved in citrate buffer (0. 2 M, pH 5. 0) and stored 12 hours in the frozen state or at 15 C. Precipitates dissolved in Rila buffer, when frozen for 12 hours, lost almost all activity, while those precipitates stored overnight at 15 C and assayed in the presence of salt-containing gelatin exhibited the greatest activity. Gelatinase dissolved in Rila buffer, stored at 15 C and assayed in gelatin substrate without salts

had approximately the same activity as Rila dissolved, frozen gelatinase.

As a result of these experiments, the subsequent 35 - 50 percent ammonium sulfate saturated precipitates were maintained and assayed in salt containing solutions with storage at 15 C.

Observations were made on a temperature range of 8 C to 50 C employing fractionated gelatinase. Optimal activity occurred at 33 C (Figure 6).

A sharp optimal pH of about 9. 0 was noted for fractionated gelatinase activity (Figure 7) when a pH range of pH 6. 0 to pH 11. 0 was studied. Activity of gelatinase nearly doubled between pH 8. 5 and pH 9. 0 with an equally sharp drop in activity from pH 9. 0 to pH 11. 0. The sharp peak at pH 9. 0 was further confirmed by other experiments with narrower pH values about the pH 9. 0 value.

Figure 8 shows the results obtained when the enzyme was assayed in gelatin substrate prepared with distilled water or varying Rila Marine salt concentrations up to 15 percent. Since the enzyme itself contained some Rila Marine salts, the salt concentration of the gelatin substrate prepared with distilled water was actually equivalent to 0. 4 percent salt content. Consequently all substrate salt concentrations were 0. 4 percent greater during the assay than in the prepared concentrations.

The greatest activity was observed with substrate prepared

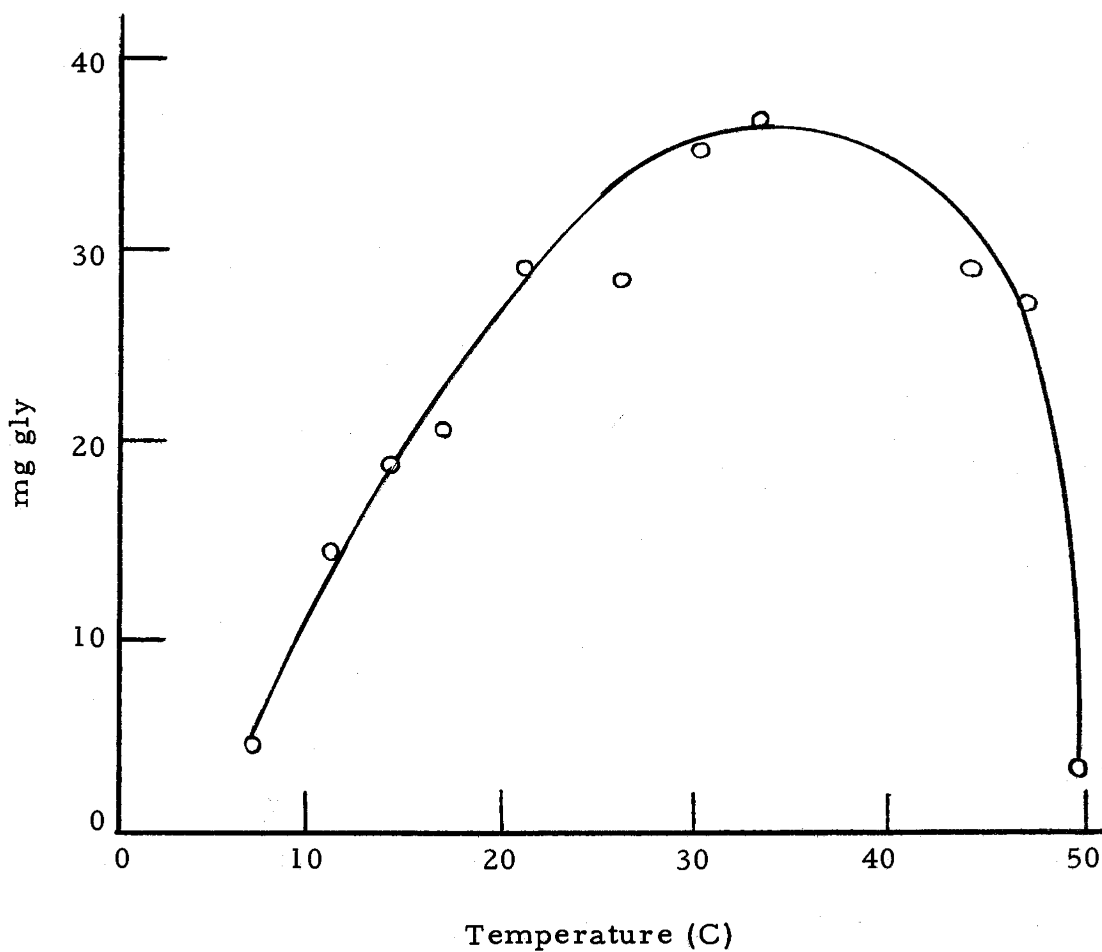

Figure 6. The effect of temperature on activity of ammonium sulfate fractionated gelatinase. The reaction mixture contained 2. 3 mg gelatinase (protein), 25 mg gelatin, and 132 mg Rila Marine salts in 6 ml, pH 7. 8. Incubation time was 24 hours in the polythermostat. The results are the average of 3 tubes.

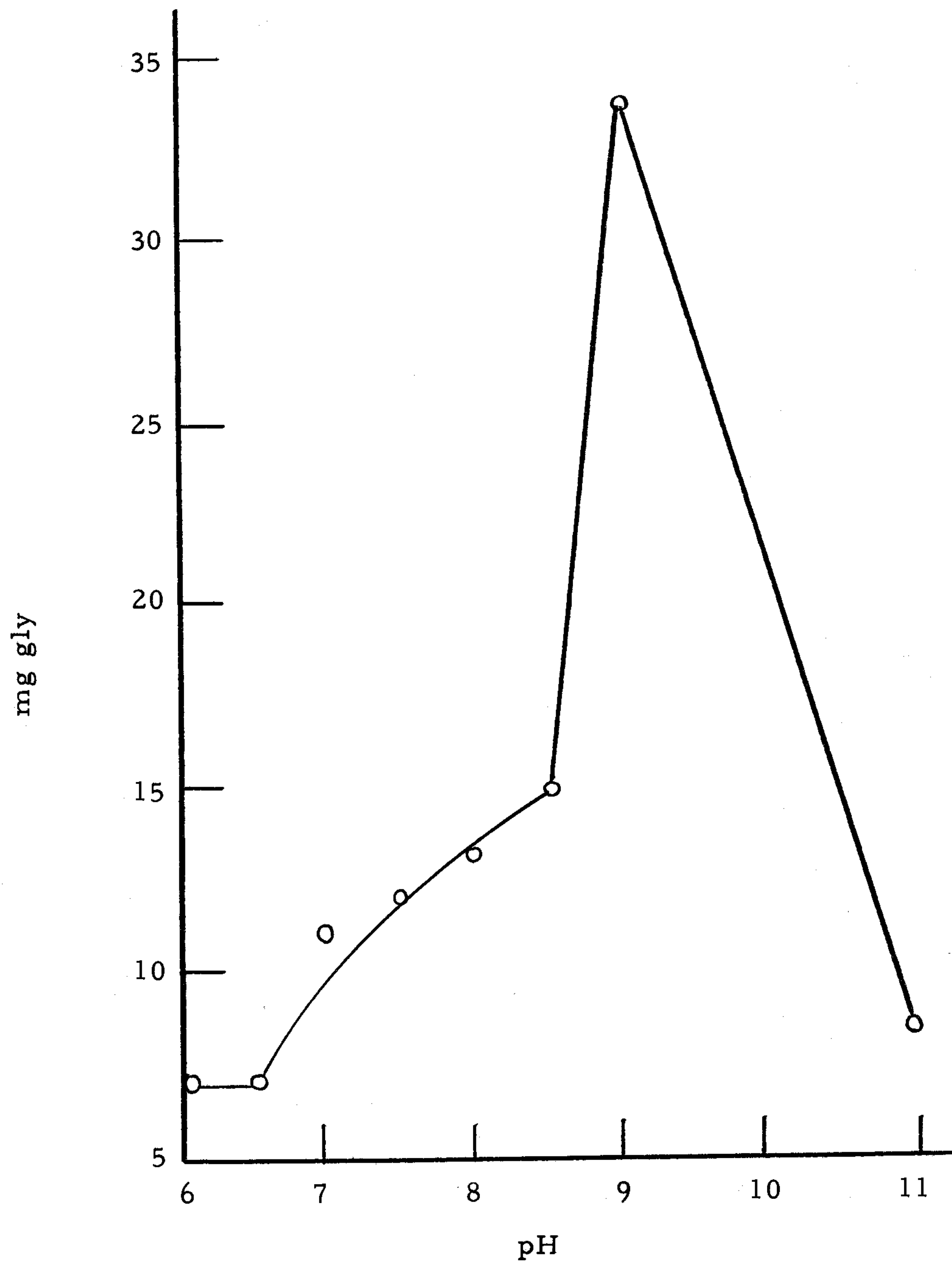

Figure 7. The effect of pH on activity of ammonium sulfate fractionated gelatinase. The reaction mixture contained 2.1 mg gelatinase (protein), 25 mg gelatin and 132 mg Rila Marine salts in 6 ml at the indicated pH values. Incubation was at 15 C for 24 hours.

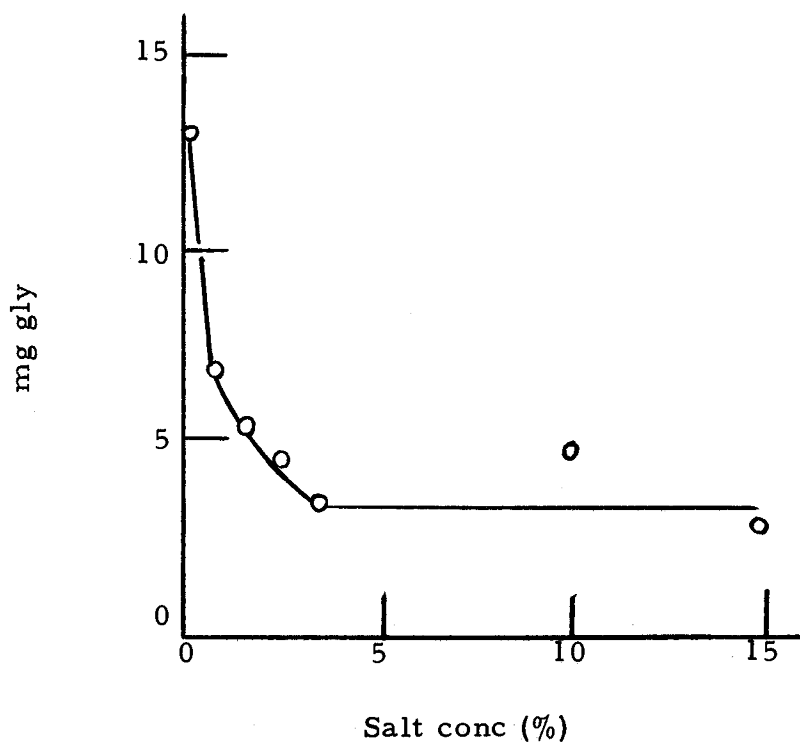

Figure 8. The effect of salt concentration on ammonium sulfate fractionated gelatinase activity. The reaction mixture consisted of 2.8 mg gelatinase (protein) and 25 mg gelatin in 6 ml, pH 7.8, with Rila Marine salts varying from 4 to 759 mg (0.4 to 15.4 percent). Incubation was for 24 hours at 15 C.

with no salts. Activity decreased to about 3.6 percent (equivalent to 100 percent sea water). Increasing salt concentrations above 3.6 percent caused little decrease in activity.

Partially Purified Gelatinase Studies

As can be seen in Table 2, activity was present only in the second void volume recovered during chromatography of the ammonium sulfate fractionated gelatinase on Sephadex G-200 gel indicating a gelatinase molecular weight of more than two million. The third void volume contained the most protein determined by the method of Warburg and Christian (45), with very little protein occurring after the twelfth void volume.

The eluent pattern obtained during the column chromatography is in Figure 9. The eluent pattern contains two peaks of 254 mμ absorbing material (about 15 ml and 50 ml) with very little absorbing material after 90 ml.

As Table 3 illustrates, a four-fold increase in specific activity was obtained between supernatant and fractionated gelatinase and a 6.5-fold activity increase was obtained between fractionated and purified gelatinase. A 28-fold increase in specific activity resulted with the entire process.

The surface denaturation of the enzyme was determined by an assay using fractionated gelatinase that had been shaken for two

Table 2. The activity of eluent recovered during chromatography of ammonium sulfate fractionated gelatinase on a Sephadex G-200 gel column*

Void Volume	Protein (mg/ml)	Protein/assay (mg)	Mg Glycine Produced
1	---	---	---
2	.338	.042	4.2
3	.633	.079	---
4	.611	.076	---
5	.413	.052	---
6	.128	.016	---
7	.067	.009	---
8	.044	.006	---
9	.039	.005	---
10	.031	.004	---
11	.024	.003	---
12	.017	.002	---

*Reaction mixtures contained 132 mg Rila Marine salts, 25 mg gelatin, and protein as listed above in 6 ml, pH 8.0. Incubation concentrations of the void volumes were as listed above and were determined by the method of Warburg and Christian (45). Incubation was at room temperature for four hours. The column (8.5 cm by 2.5 cm diameter), packed with Sephadex G-200 gel and Rila buffer, pH 7.9, as the eluent, had a void volume of 8.8 ml.

Table 3. Activity of the different gelatinase preparations*

Gelatinase Preparation	Protein/assay (mg)	Units
supernatant gelatinase	1.44	1.6
fractionated gelatinase	0.38	6.6
purified gelatinase	0.06	43.8

*The reaction mixtures contained 132 mg Rila Marine salts, 25 mg gelatin, and the indicated amounts of protein in 6 ml, pH 8.0. Incubation was for one hour at 40 C. The unit is defined as mg glycine produced/mg gelatinase/hour.

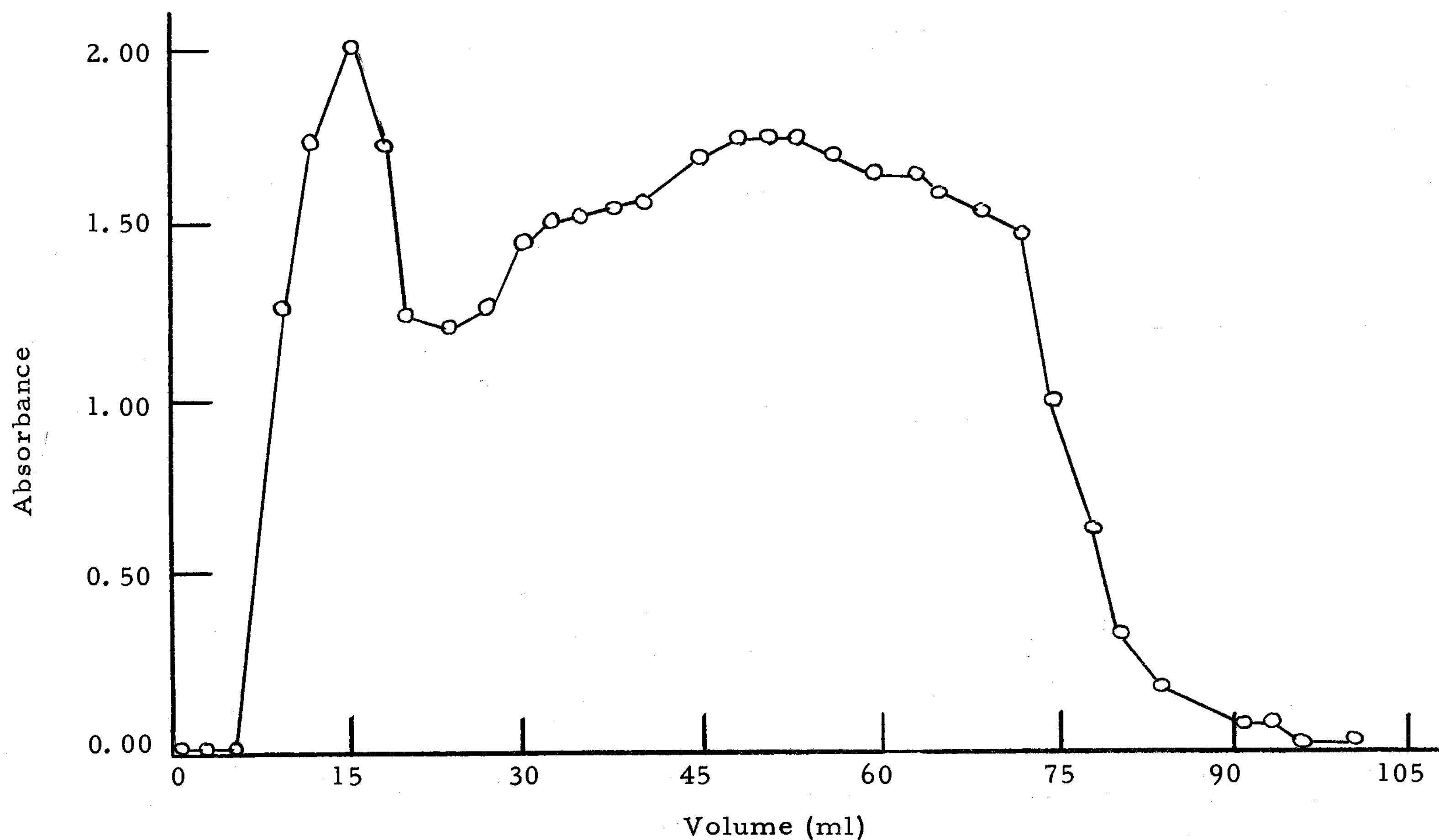

Figure 9. Chromatography of ammonium sulfate fractionated gelatinase on a column (8 cm by 2.5 cm diameter, void volume 8.0 ml) of Sephadex G-200 gel in Rila buffer, pH 7.9, at room temperature. The gelatinase was an appropriate amount of the 35 - 50 percent saturated fraction of the supernatant dissolved in 3 ml Rila buffer.

minutes on a Vortex Junior mixer prior to assay. The control was an unshaken portion of the same gelatinase preparation. No significant difference was noted between the two systems.

Over a temperature range of 15 C to 51 C, assay of purified gelatinase (Figure 10) showed little activity increase between 15 C and 20 C with increasing activity up to an optimum for purified gelatinase activity at 40 C. Purified gelatinase assays were run at this temperature optimum.

Figure 11 graphs the results obtained when gelatinase concentrations of 0. 025 mg to 0. 200 mg protein per ml were assayed. A gelatinase concentration of 0. 07 mg to 0. 08 mg per ml provided the most activity although activity was still present at a concentration of 0. 025 mg gelatinase per ml (the most dilute concentration assayed).

Substrate concentration studies were performed employing 0. 08 mg gelatinase per ml in each reaction. The enzyme system was found to be saturated at 0. 5 percent substrate gelatin (Figure 12). Therefore in none of the subsequent experiments does the substrate become limiting.

Figure 13 shows the rate of glycine production due to hydrolysis at 5 C and 40 C. The rate of glycine production is much lower at the organism's environmental temperature than at higher temperatures. The optimal temperature for purified gelatinase activity is 40 C.

Purified gelatinase, assayed over the range of pH 7. 5 to

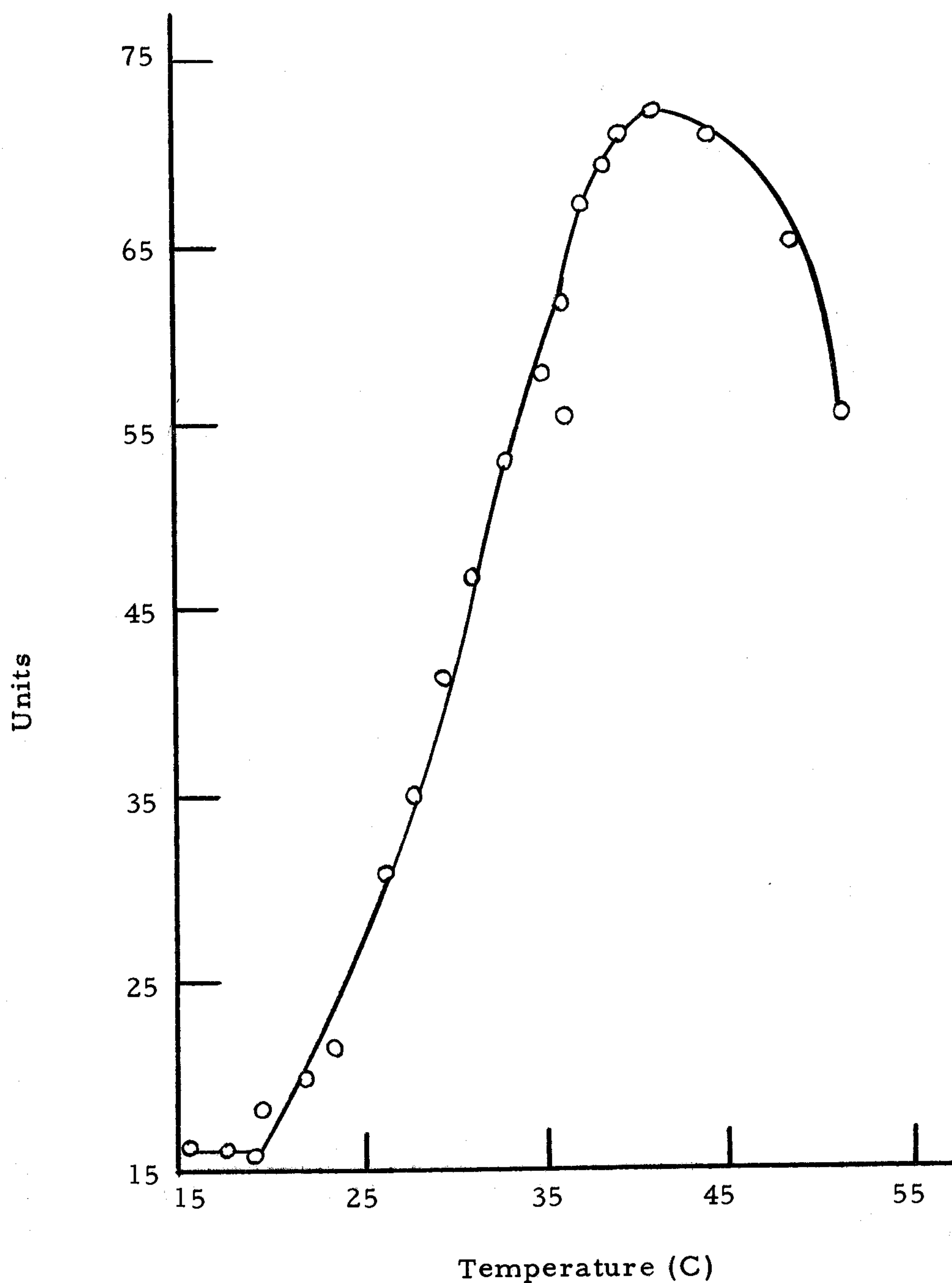

Figure 10. The effect of temperature on purified gelatinase activity. The reaction mixture contained 132 mg Rila Marine salts, 25 mg gelatin, and 0.39 mg gelatinase (protein) in 6 ml, pH 8.0. Incubation was for 1 hour in the polythermostat. The results are the average of three tubes. The unit is mg glycine produced/mg gelatinase/hour.

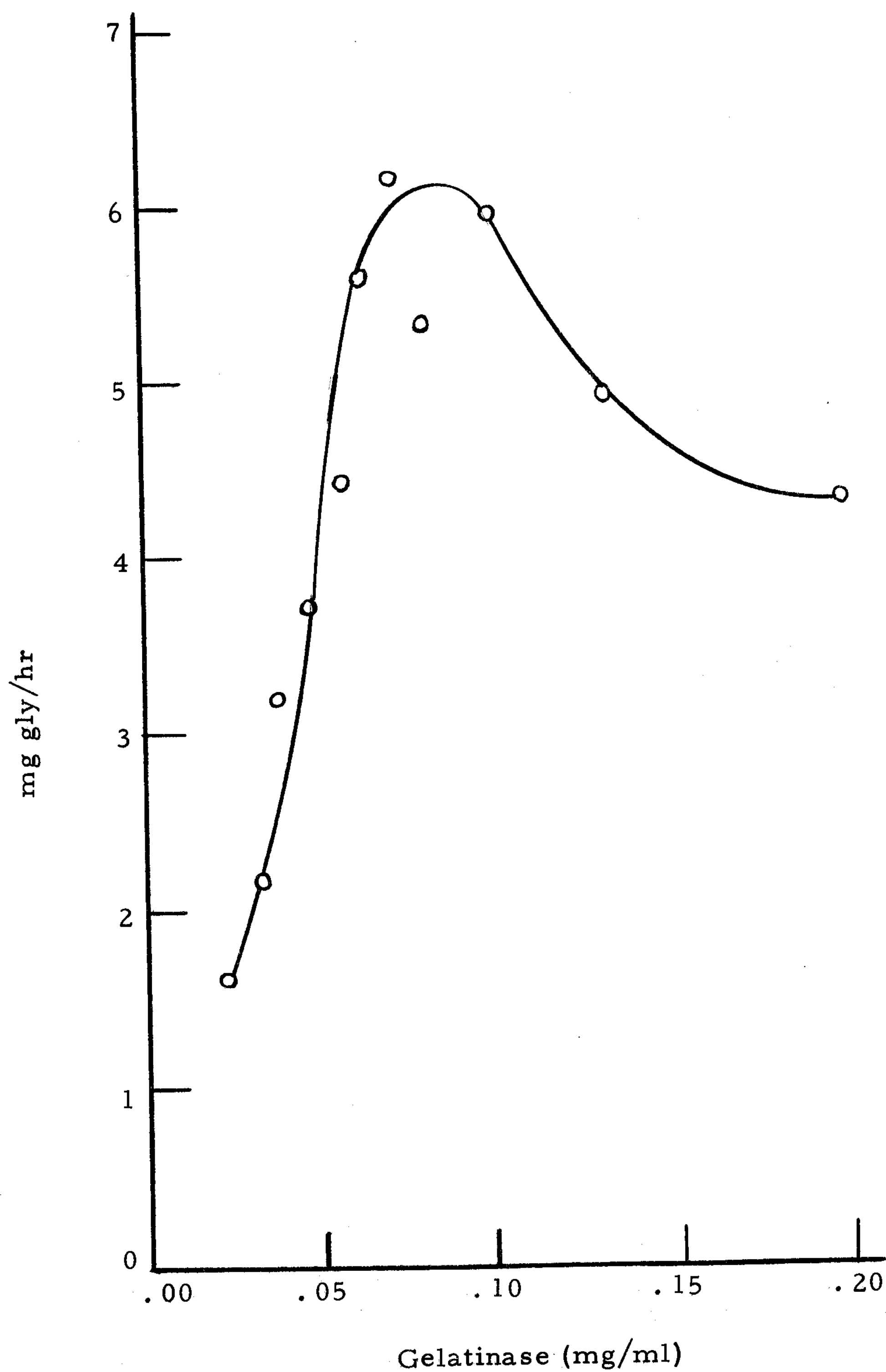

Figure 11. The activity of various concentrations of purified gelatinase. The reaction mixture contained 132 mg Rila Marine salts, 25 mg gelatin, and 0.12 mg to 10.00 mg gelatinase (protein) in 6 ml, pH 8.0. Incubation was for 1 hour at 40 C.

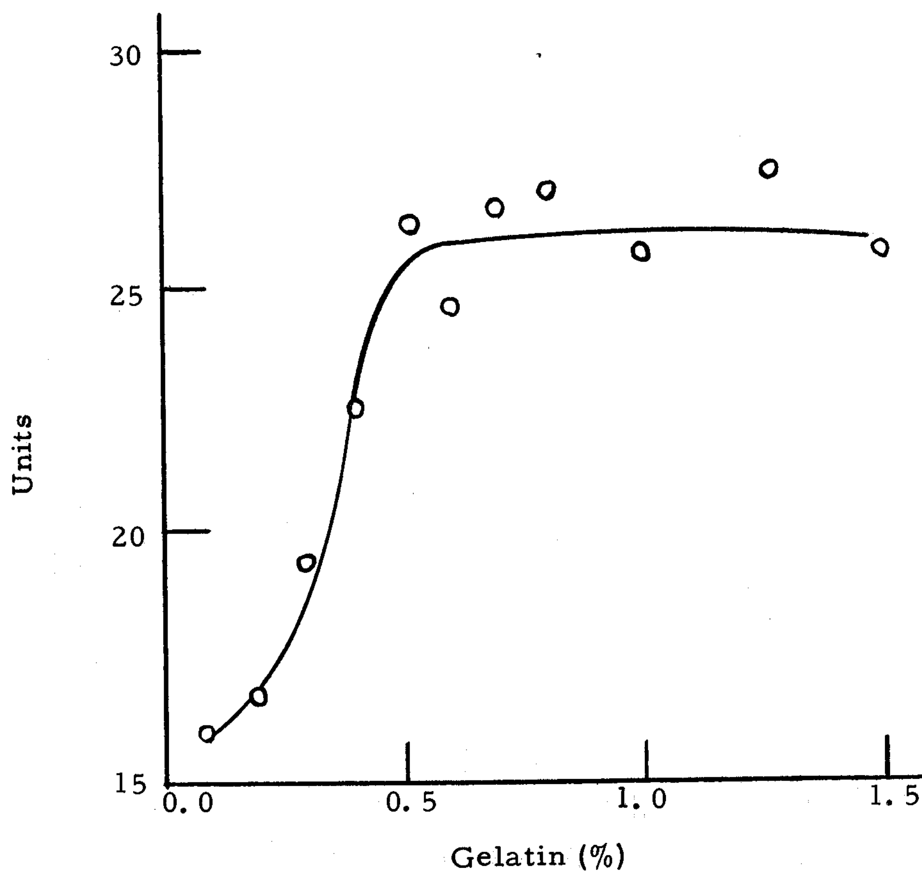

Figure 12. Purified gelatinase activity at various substrate gelatin concentrations. Reaction mixtures contained 132 mg Rila Marine salts, 0. 40 mg gelatinase, and 5 mg to 75 mg gelatin (0. 1 to 1. 5 percent concentration) in 6 ml, pH 8. 0. Incubation was for 1 hour at 40 C. The unit is mg glycine produced/mg gelatinase/hour.

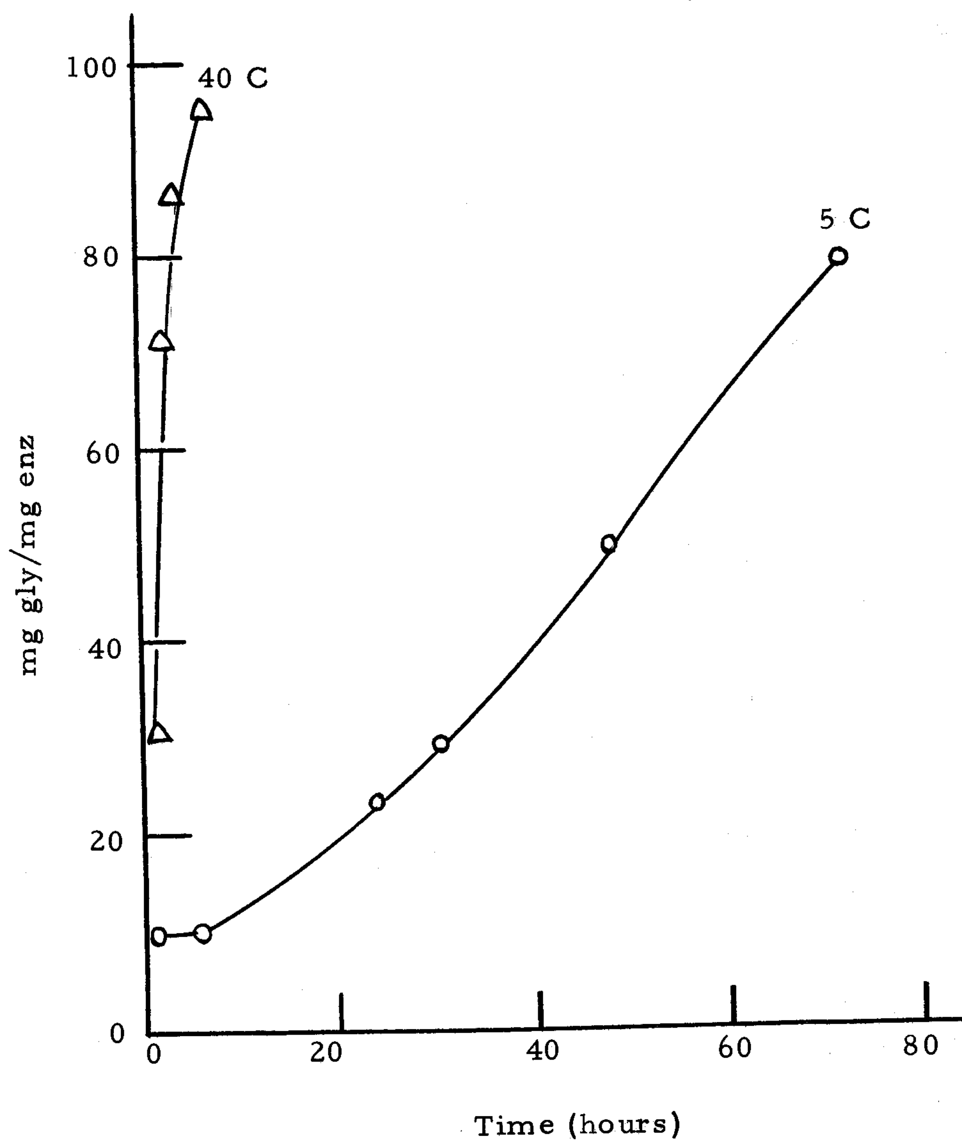

Figure 13. Rate of hydrolysis by purified gelatinase at 5 C and 40 C. The reaction mixtures contained 132 mg Rila Marine salts, 25 mg gelatin, and .52 mg gelatinase (protein) in 6 ml, pH 8.0. Incubation was at 5 C or 40 C for the indicated time intervals. The unit is mg glycine produced/mg gelatinase/hour.

pH 9.5, had optimum activity at pH 9.3 (Figure 14).

A curve similar to the one obtained with fractionated gelatinase (Figure 8) occurred when purified gelatinase was assayed with substrate differing in salt content (Figure 15). Again the optimum activity was found in the absence of salt. Little change in activity resulted when the salt concentration was greater than 3.6 percent.

A dialyzed gelatinase, free of salts, was used to determine activity in the absence of sea water or salts. The dialyzed gelatinase was assayed with gelatin substrate and gelatin substrate prepared without Rila Marine salts. In both cases no gelatinase activity was observed.

Pressure studies on purified gelatinase at room temperature and 40 C (optimum temperature for purified gelatinase activity) over the pressure range of 1 to 600 atm were carried out. As with MP-41 pressure studies (Figure 5), the higher temperature showed greater activity (Figure 16). The activity at 40 C was about twice as great as that at 25 C. A slight decrease in activity occurred at 100 atm at 25 C. Further pressure increases caused no further activity change. At 40 C there was a very slight continuous activity decrease from 1 to 600 atm with no marked changes in activity observable (Figure 16). No significant activity occurred during the pressurization period.

Purified gelatinase, when assayed in the presence of ammonium sulfate, exhibits increased activity (Table 4). A two-fold activity

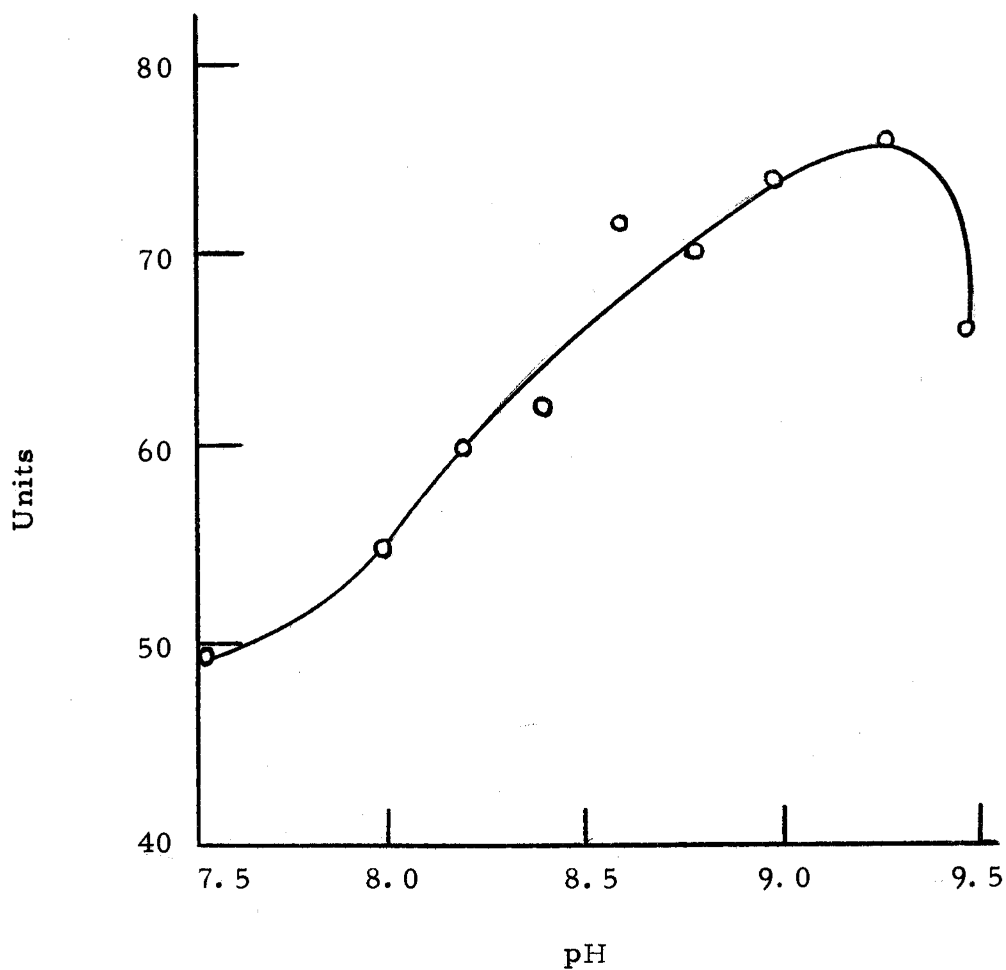

Figure 14. The effect of pH on purified gelatinase activity. The reaction mixture contained 132 mg Rila Marine salts, 25 mg gelatin, and . 35 mg gelatinase (protein) in 6 ml at the indicated pH values. Incubation was at 40 C for 1 hour. The unit is mg glycine produced/mg gelatinase/hour.

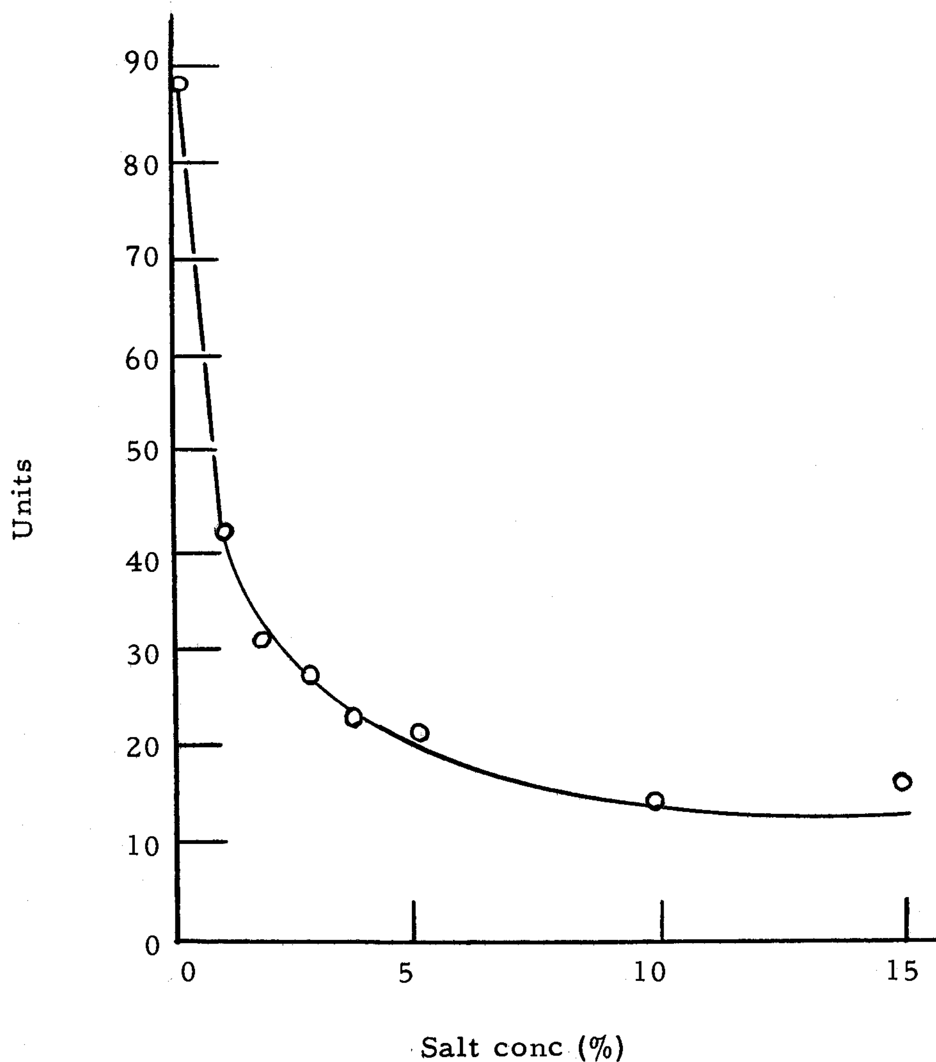

Figure 15. The effect of salt concentration on activity of purified gelatinase. The reaction mixture contained 0. 35 mg protein (gelatinase), 25 mg gelatin and 4 mg to 759 mg Rila Marine salts (0. 4 to 15.4 percent) in 6 ml, pH 8. 0. Incubation was for 1 hour at 40 C. The unit is mg glycine produced/mg gelatinase/hour.

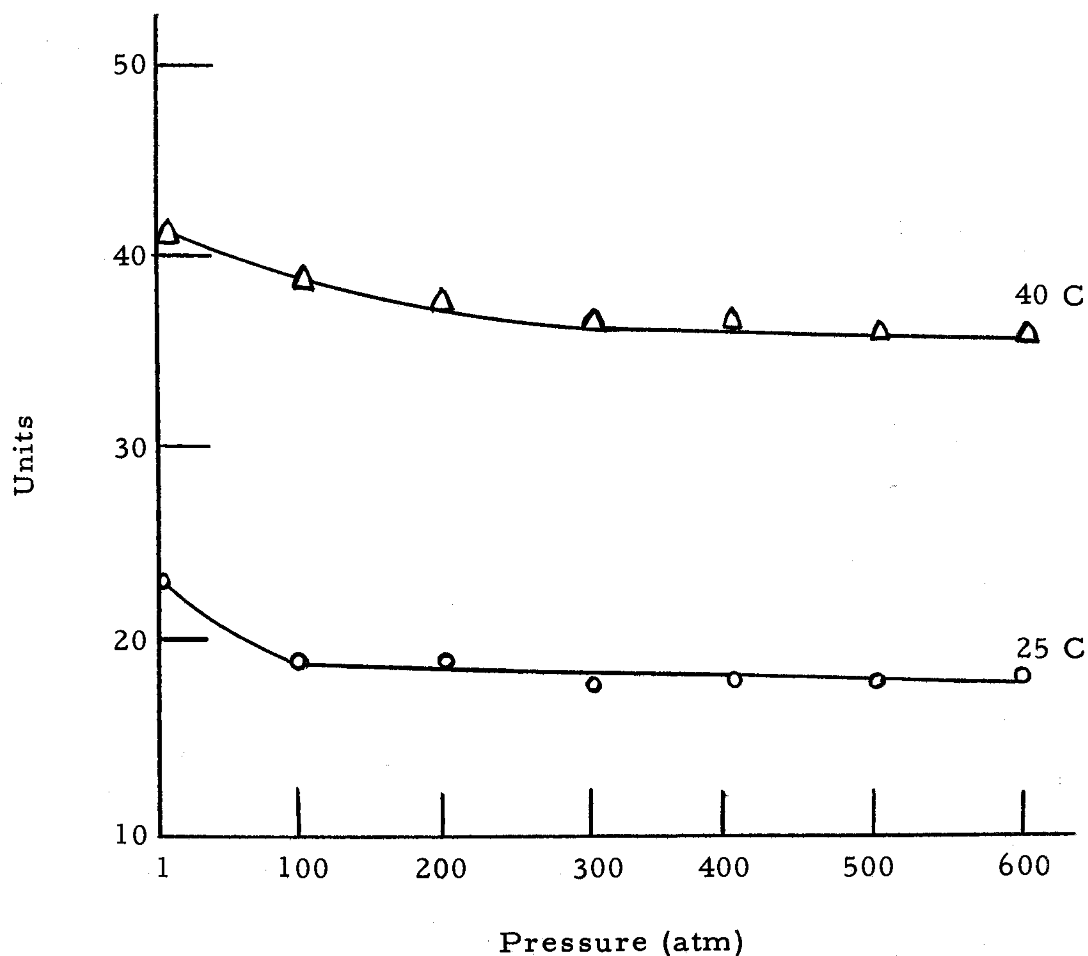

Figure 16. Pressure effects on purified gelatinase activity at 25 C and 40 C. The reaction mixture contained 80 mg Rila Marine salts, 15 mg gelatin, and 0.35 mg gelatinase (protein) in 4 ml, pH 8.0. The reaction mixture was brought to 6 ml with 2 ml Rila buffer following incubation in 10 mm by 38 mm test tubes within pressure cylinders for 1 hour at the indicated pressures and 40 C or 25 C. The unit is mg glycine produced/mg gelatinase/hour.

increase was observed when gelatinase containing 313 mg ammonium sulfate per ml (equivalent to 50 percent ammonium sulfate saturation) was used instead of regular purified gelatinase in the ninhydrin assay.

Table 4. The effect of ammonium sulfate on purified gelatinase activity*

Gelatinase Preparation	Units
purified gelatinase	43. 8
purified gelatinase plus 1. 56 g ammonium sulfate	97. 8

*The reaction mixture contained 132 mg Rila Marine salts, 25 mg gelatin, and 0. 30 mg gelatinase (protein) in 6 ml, pH 8. 0. The concentration of ammonium sulfate was equivalent to 50 percent saturation. Incubation was one hour at 40 C. The unit is mg glycine produced/mg gelatinase/hour.

DISCUSSION

Preliminary studies were performed to determine the optimum temperature and pH for growth of MP-41. This organism, according to the definition of Stokes (40), is an obligate marine psychrophile growing optimally at 18 C with no growth above 25 C. At 0 C colonies were visible on gelatin media within one week. Data by Colwell (Appendix) supports these temperature observations. However, this optimum temperature for growth does not coincide with its original environmental temperature (3. 24 C).

Growth of MP-41 occurred within the pH range found in the oceans (pH 7. 5 to pH 8. 5). Gelatinase produced by the cells was also active in this range. At pH 7. 6 or lower cellular gelatinase activity did not occur.

These values are higher than those for many other psychrophilic proteinases studied (26, 36, 43) but Peterson (34) using ammonium sulfate fractions of culture filtrate from the psychrophile, _Pseudomonas fluorescens_, obtained optimum activity at pH 8. 8.

Whole cell gelatinase assays were performed with washed cells grown in a gelatin medium. A short incubation time interval was employed to prevent cell proliferation and to thereby measure gelatinase production and subsequent activity rather than activity resulting from increased cell numbers. The ability to produce the

exoenzyme was maximal at the highest temperature employed in the study (24 C) which was also the organism's maximum growth temperature. This temperature does not coincide with the organism's optimum temperature for growth or its environmental temperature. It should be mentioned that gelatinase synthesis does take place at temperatures close to the organism's environmental temperature.

If cell-free extracts of twice-washed cells were assayed for their gelatinase activity, very little gelatinase activity could be demonstrated. Further, cells grown in the absence of a gelatin substrate in the medium would not produce any gelatinase.

The ability of cells to produce gelatinase is also influenced by both temperature and pressure. At 15 C the effects of pressure are more pronounced between 1 and 600 atm showing that reduced pressures decreased the gelatinase production and activity. Although less gelatinase is produced at 10 C than 15 C, the effect of 1, 100, and 200 atm is not pronounced. A sudden decrease occurs between 200 and 300 atm, possibly reflecting a sudden change in the conformation of cellular constituents. This conformational change may result in either less gelatinase production or a loss of activity in the gelatinase itself.

Working with fractionated gelatinase the temperature of optimum activity occurred at approximately 33 C demonstrating that in the crude enzyme preparation the activity is well beyond the maximum

growth temperature of the organism. A sharp pH optimum occurred at pH 9.0 but marked activity was found between pH 7.5 and pH 8.5, the approximate pH of the sea.

Studies with partially purified gelatinase indicated that the optimum temperature for gelatinase activity was 40 C. Although the organism that produces this enzyme can be termed an obligate psychrophile, the enzyme is not "psychrophilic" in nature. Similarly, using culture supernatants from proteolytic psychrophiles, other researchers (26, 36, 43) have found optimal activities occurring from 30 C to 50 C indicating that the proteolytic enzymes under study were not thermolabile.

If one assumes that the evolution of microorganisms was such that mesophilic bacteria were the ancestors of the psychrophilic bacteria, then this organism has not completely evolved to produce a "psychrophilic" gelatinase even though many of its cellular enzymes may be psychrophilic in nature. Ingraham and Bailey (16), studying dehydrogenases, found no significant difference in the effect of temperature on mesophiles and psychrophiles.

From a more practical viewpoint, the above situation points out that proteolysis of marine products could occur long after the death of the psychrophilic bacterium. This may have important economic significance.

When a comparison of activity is made at 40 C and 5 C, the

rate at 40 C is extremely rapid while the rate at 5 C is extremely low.

When the system is subjected to hydrostatic pressure rate of hydrolysis is decreased with increasing pressures. A large difference occurs between the amount of activity at 25 C and 40 C at 1 atm. Pressure application between 1 and 600 atm does not produce a drastic change in activity. The enzyme apparently is more sensitive to a change in temperature than a change in pressure. The rate of hydrolysis at 5 C under pressure was not practical in the laboratory due to the time element.

From a theoretical viewpoint, the function of temperature and pressure go hand in hand, especially when one takes into consideration the Ideal Gas Law ($PV = nRT$). The studies of protein denaturation provide a good insight into changes that take place.

Enzyme (protein) denaturation has been defined by Kauzmann (19) as a process (or a sequence of processes) in which the spatial arrangement of the polypeptide chains within the molecule is changed from that typical to the native protein to a more disordered arrangement. This disordered arrangement results in volume changes (ΔV) in the protein. Temperature increases, alkaline pH, and the presence of certain compounds (alcohol, etc.) favor protein denaturation while high hydrostatic pressure retards or reverses denaturation (10). Low or moderate pressures often inhibit denatunaturation or enhance stability (18). The active state may occur in the native or denatured

form depending on the enzyme system in question.

This enzyme resembles the aspartase system studied by Haight and Morita (13). An increase in temperature (ΔV increase) exposes more reactive sites resulting in increased activity which is reversed or retarded by pressure.

Johnson et al. (18) partially attributed a decrease in ΔV to ionization. Increased internal ionization as a result of less interference from other materials (partially purified gelatinase) could cause the ΔV decrease requiring higher temperatures to oppose the ΔV change, thus raising the temperature for denaturation or optimal activity.

Therefore, as can be seen by this study on gelatinase, the effects of reduced temperature and increased pressure are additive. Both bring about a ΔV decrease, hence a lower rate of gelatinase activity is observed at low temperatures and increased hydrostatic pressures.

Attempts to extrapolate laboratory studies to the natural environment must be done with great care. However, there are a few important points this investigation brings out that can be related to the natural environment. This inducible exoenzyme produced by the psychrophile, MP-41 functions well at the salinity of sea water. Activity can be noted at low temperature as well as increased pressure. Vigorous agitation of the enzyme (as might be found in the

natural conditions) does not destroy the enzyme and, at the low concentration studied in this investigation, the enzyme is active. However, it is recognized that the concentration employed in this study would still be rather high for open ocean water.

The greatest concentration of bacteria have been shown by ZoBell and Anderson (52) to occur at the mud-water interface. If an investigation of free enzymes in the sea were to be initiated, the mud-water interface should be the ideal location to look for these free enzymes. The salting out effect of sea water and the presence of dead organisms that have settled to the bottom should tend to concentrate free enzymes at the mud-water interface.

Although the existence of free enzymes in oceanic waters remains problematical, this study has investigated the effects of various parameters (temperature, pressure, pH, salinity) on the action of the exoenzyme, gelatinase. These studies can, in part, be related to the natural environment.

SUMMARY

Partial purification of the inducible exoenzyme, gelatinase, from Marine Psychrophile 41 (MP-41) an obligately psychrophilic marine vibrio, was accomplished by column chromatography on Sephadex G-200 gel of the 35 - 50 percent ammonium sulfate fraction from the culture filtrate.

This organism, whose optimal temperature and pH for growth were shown to be 18 C and pH 8. 0 to pH 8. 3, displayed maximal gelatinase activity at its maximum growth temperature (24 C) and at pH 8. 8. The 35 - 50 percent ammonium sulfate fraction showed optimal activity at 33 C and pH 9. 0.

Twenty eight-fold purified gelatinase was optimally activity at 40 C and pH 9. 3. This partially purified gelatinase was saturated in an 0. 5 percent gelatin substrate but sill active in an 0. 1 percent gelatin substrate. Enzyme activity was optimal at 0.07 to 0.08 mg per ml concentrations with activity observed at the minimal concentration studied, 0. 025 mg per ml. The rate of hydrolysis at 5 C was shown to be considerably lower than at 40 C.

The fractionated and purified gelatinases displayed optimal activity in an 0. 4 percent salt concentration (the lowest studied) with decreasing activity as the concentration increased. Salt-free systems demonstrated no activity. Ammonium sulfate caused a two-fold

increase in purified gelatinase activity.

Pressure increases decreased the activity of cells and purified gelatinase.

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