PURIFICATION AND CHARACTERIZATION OF PROTEOLYTIC ENZYMES FROM *BACTEROIDES AMYLOPHILUS* H-18

by

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ABSTRACT

This study purposes to examine extracellular proteases of the anaerobic rumen bacterium, Bacteroides amylophilus H-18. An enzyme was isolated and purified from 29 litres of 23 hr cell-free culture supernatant using DEAE Sephadex A-50, Sephadex G-200 and isoelectrofocusing techniques. Although proteolytic activity in the supernatant had a peak of activity at pH 6.7, there was activity at pH values from 4.5 to 11.5. Therefore, an attempt was made to purify the pH 6.7 activity and to follow the activity at other pH values as an index of purity. It was found that separation of the activities at different pH values was not achieved, even though the enzyme was purified 1265 times. Gel filtration of this purified material revealed the presence of two proteases, one of 60,000 and the other of 30,000 molecular weight. Since these enzymes were otherwise identical, they could have represented the monomeric and dimeric forms of a single protein. If the protease of 30,000 molecular weight was separated and resubjected to gel filtration, protease activity of molecular weight 60,000 reappeared. Ultracentrifugation of the 30,000 molecular weight protease demonstrated only one component. Therefore, if the two forms were in equilibrium, it appeared that the dimer was the more stable form of the enzyme. The purified protease did not contain cysteine, so that any tertiary structure in the enzyme could not involve disulfide bridges. All attempts to dissociate the dimeric into the monomeric form were unsuccessful.

Examination of the inhibition of Nα benzoyl-L-arginine methyl ester esterase and protease activities with Nα tosyl-L-chloromethane revealed a complete inhibition of esterase activity at pH 8.0 but only a 30% inhibition of protease activity at the same pH, suggesting that more than one enzyme was responsible for the proteolytic activity exhibited by the purified enzyme. Because it was not possible to achieve separation of proteolytic activities at different pH values after a 1265 times purification, it must be assumed that if there are actually different proteases present they must have very similar structures.

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INTRODUCTION

Bacteroides amylophilus H-18, a Gram-negative proteolytic organism, was isolated by Blackburn and Hobson (1962) from the rumen of a sheep using strict anaerobic conditions. Isolation of such an organism was considered unusual, since, with the exception of members of the genera Pseudomonas and Vibrio (Pollock, 1962), most bacteria which produce exoenzymes are Gram-positive. Protease production in B. amylophilus H-18 is not subject to either product repression or induction, nor is a general metabolic repression demonstrable (Blackburn, 1968 a). The presence of 0.1% (w/v) tryptose in the medium did decrease the lag period, probably by stabilizing the E_h value; however, additional nutrients did not affect either growth or protease production.

Protease activity from B. amylophilus H-18 is of interest as its function is unknown. Hobson et al. (1968) observed that some 93% of the cell nitrogen could be accounted for by ammonia-^{15}N disappearance in media with and without additional identified sources of nitrogen. This confirmed the findings of Abou Akkada and Blackburn (1963) that B. amylophilus H-18 produced relatively small quantities of free amino acids from protein, and this was in common with other proteolytic rumen bacteria which appeared to utilize ammonia in preference to preformed amino acids and peptides. The function of the protease from B. amylophilus H-18 may be associated with muco-peptide synthesis. Whitaker (1965) purified an α and β-lytic protease from Sorangium Sp. which hydrolyzed

muco-peptides from the cell walls of Arthrobacter globiformis and Micrococcus lysodeikticus. Another possible function of the proteolytic activity may be the liberation of exocellular amylases. B. amylophilus H-18 has an absolute requirement for maltose or for 1,4-linked glucose polymers (Blackburn and Hobson, 1962) and the protease may assist the release of the amylase.

Blackburn (1968 b) showed that the protease liberated by sonic disruption of log phase cells of B. amylophilus H-18 was excluded from G-200 Sephadex (exclusion limits 200,000 M.W.) and could not be easily purified. In this respect it resembled the penicillinase of Bacillus licheniformis which, when liberated by lysozyme treatment, appeared to be bound to membrane fragments (Lampen, et al 1968). Therefore, it was necessary to determine whether the protease liberated in the stationary phase was of small molecular weight, indicating preferential liberation, or whether it was of large molecular weight and presumably membrane or particle bound. If the latter were true, there would be no advantage in attempting its purification as similar material could more conveniently be prepared from the whole, log-phase cells.

Blackburn (1968 b) suggested the presence of two proteases on the basis of different esterase to protease ratios. It was found that Mickle disintegration of lyophilized cells suspended in water gave good yields of protease with low p-toluenesulphonyl-L-arginine methyl

ester (TAME) esterase and casein-precipitating activities. The TAME-esterase had a pH optimum at 8.0 but the protease had a broad optimum with minor peaks at pH 6.5 and 8.0.

There is a complete inhibition of esterase activity with an 87% inhibition of protease activity by di-isopropylphosphofluoridate (DFP) (Blackburn, 1968 b). This is similar to the family of Sorangium Sp. of serine proteases of Whitaker (1967) who showed the lytic protease to be readily inhibited by DFP while the lytic enzyme was not.

Since the protease from B. amylophilus H-18 showed a variety of different pH optima, ion-exchange chromatography on DEAE Sephadex A-50, gel filtration through Sephadex G-200 and isoelectrofocusing were used in an attempt to obtain separation of proteases.

The main purpose of this project was the purification of pH 6.7 proteolytic activity and the study of its properties to determine its homogeneity. The situation of other proteolytic activities being purified similtaneously is coincidental.

Disulfide bridges may possibly be involved in the maintenance of the tertiary structure of the protease. It was therefore of interest to measure the cysteine content in the protease. Pollock (1962) demonstrated a low cysteine content in extracellular bacterial proteins. More specifically, he found an exceptionally low content of cysteine in a variety of bacterial proteases and extracellular enzymes.

MATERIALS

Sephadex G-200, DEAE Sephadex A-50 and SE Sephadex C-50 were purchased from Pharmacia, Uppsala, Sweden. The media constituents and chemicals were of reagent grade and obtained from Fisher Scientific Co., Fairlawn, New Jersey; soluble starch was from The British Drug House, Poole, England. Bovine serum albumin and Nα tosyl-L-chloromethane HCl and Nα benzoyl-L-arginine methyl ester from Calbiochem, Los Angeles, California.

The LKB fraction collector, Uvicord (0.3 cm light path), and the 8100 Ampholine Electrofocusing Equipment were purchased from LKB Produkter AB, Stockholm, Bromma, Sweden. Other equipment included a Servall Type SS-34 and GSA centrifuge; a KSB:R Servall continuous flow adaptor from Servall, Norwalk, Connecticut; MSE centrifuge, London; Diaflo Model 50 ultrafiltration cell from Amicon Co., Lexington, Mass; Spectronic 20 spectrophotometer from Bausch and Lomb, Rochester, N.Y.; Beckman Model 120 amino acid analyzer and Beckman Model E analytical ultracentrifuge from Beckman Instruments, Palo Alto, California. A Radiometer Type PHM 28 was obtained from Radiometer Ltd., Copenhagen, Denmark.

METHODS

Determination of Protein:

Protein concentration was measured by the method of Lowry et al (1951) using bovine serum albumin (BSA) as the standard.

Determination of Protease Activity:

The assay procedure was a modification of the McDonald and Chen (1965) method. The reaction mixture contained 0.2 ml of enzyme solution and 1.8 ml of a 2.0% (w/v) solution of casein in 0.01% merthiolate, 0.1 M phosphate buffer. The pH of the casein solution was 6.7. Where necessary, reactions were run at other pH values by making the casein up in various buffers and adjusting the pH with either NaOH or HCl. The mixture was incubated at 38 C for varying lengths of time, depending on the concentration of proteolytic activity within a given sample. At the end of the incubation period, 2.0 ml of 0.72 N trichloroacetic acid (TCA) were added and mixed well to precipitate all the protein. The precipitated digest was allowed to stand at room temperature for 10 min. After centrifuging at 1000 xg for 10 min, 1.0 ml of the TCA soluble portion was removed with a pipette and added to 5.0 ml of copper solution. The copper solution was prepared by adding 1.0 ml of 0.5% $CuSO_4$ in 1.0% sodium citrate to 50 ml of 1.0 N NaOH in 2.0% Na_2CO_3. After 15 min incubation at 38 C, 0.5 ml of 1.0 N Folin

Reagent was added and mixed in immediately using a Vortex-Genie mixer. The tubes were then incubated at 38 C for 30 min and the extinctions were read at 700 mμ on a Spectronic 20. The extinction at zero time (where TCA was added before incubation) was subtracted from the extinction of the test and the corrected value compared with a standard BSA curve (100 μg BSA giving an E_{700} of 0.32). A unit of proteolytic activity was defined as the amount of enzyme which under standard conditions would solubilize the equivalent of 1.0 μg BSA in one min. There was a linear relationship between the time of incubation and the amount of casein solubilized. Specific activity was defined as units of protease activity per milligram of protein.

Determination of Amylase Activity:

One ml of enzyme was added to 1.0 ml of a 1.0% (w/v) solution of soluble starch in 0.2 M Tris(hydroxymethyl)aminomethane, 0.1 M malate. The pH of the starch solution was 6.9. After 30 min incubation at 38 C, 2.0 ml of stopper reagent were added. The stopper reagent was an alkaline solution of dinitrosalicylic acid. After the stopper reagent was added to the enzyme mixture, it was boiled for 5 min cooled and diluted with 20 ml of water. The extinctions were read at 540 mμ on a Spectronic 20. The extinction of the control, which had the stopper reagent added immediately before incubation, was subtracted from the extinction of the test sample and this corrected value was compared

to a standard maltose curve (1.0 mg maltose per ml gave an E_{540} of 0.220). A unit of amylase activity was defined as the amount of enzyme which under standard conditions would solubilize the equivalent of 1.0 milligram maltose in one min.

Determination of Esterase Activity:

Esterase activity was measured by the method of Schwert et al (1948) by using a Radiometer Type PHM 28, Radiometer Ltd., Copenhagen, Denmark. The reaction mixture contained 0.4 ml of 0.1 M ester substrate, 0.5 ml water, and 0.1 ml enzyme preparation and was incubated at 25 C under N_2. The pH of the mixture was maintained by titration with 0.1 N NaOH from a 0.5 ml micrometer syringe.

Inhibition of Proteolytic Activity with Nα tosyl-L-chloromethane(TLCM):

A 0.1 ml sample of enzyme preparation was incubated with 0.9 ml of 1×10^{-2} M TLCM at 25 C for one hr. Then protease activity was assayed at pH 5.0, 6.7, 8.0 and 11.0. Esterase activity of the treated enzyme was assayed at pH values 5.0 to 11.0.

Ultracentrifugation:

The enzyme sample was run in a synthetic boundary cell at 56,000

rev/min in 0.1 M phosphate buffer (pH 7.0); bar angle, 60 °; protein concentration, 0.3 mg/ml; pictures were taken 5 min after attaining full speed at 4 min intervals.

Bacteroides amylophilus H-18 was isolated by Blackburn and Hobson (1962) and was maintained as described by Blackburn (1964 a). The preparation and composition of the media were also described by Blackburn (1968 a). The basal medium contained (g/l): K_2HPO_4, 0.45; KH_2PO_4, 0.45; $(NH_4)_2SO_4$, 0.9; NaCl, 0.9; $MgSO_4.7\ H_2O$, 0.09; $CaCl_2$, 0.09; resazurin, 0.001; L-cysteine HCl, 0.5; $NaHCO_3$, 5.0; maltose, 3.0; and tryptose, 3.0.

Isolation of Proteases from B. amylophilus H-18.

A 32 litre stainless steel milk can containing 29 litres of growth medium (Blackburn, 1968 a) was inoculated with a one litre log phase culture of B. amylophilus H-18 and was incubated anaerobically under CO_2 for 23 hr at 38 C. Then the can and its contents were cooled rapidly with a water hose and the cells removed by continuous flow centrifugation at 8700 xg at 4 C using a Servall centrifuge.

The optimal pH for the attachment of the protease to the ion-exchanger was found to be pH 5.5. Fortunately it was not necessary to adjust the pH of the supernatant as the 23 hr culture of B. amylophilis H-18 had a pH of 5.5. DEAE Sephadex A-50 (0.2 g dry weight/100 ml) was added batchwise to the culture supernatant and CO_2

was bubbled through the suspension overnight at 4 C to ensure good mixing. After allowing the suspension to settle, the supernatant was poured off and the DEAE collected on a sintered glass filter. The DEAE was suspended in 500 ml of 0.1 M NaCl, the suspension mixed well and then centrifuged. The supernatant was poured off and stored. The DEAE was extracted in this way a total of six times. The first five extractions (total volume 2500 ml) were pooled and dialyzed against 0.05 M phosphate buffer (pH 7.0).

A DEAE Sephadex A-50 column (5.0 cm x 100 cm) was equilibrated with 0.05 M phosphate buffer (pH 7.0) and the 2500 ml of dialyzed protease passed through it. The enzyme was eluted with a linear gradient of 0.2 M to 1.0 M NaCl in the same buffer and the fractions were tested for proteolytic activity at pH 6.7. The active fractions were pooled and dialyzed against 0.05 M phosphate buffer (pH 7.0). The solution was concentrated to 70 ml with a Diaflo ultrafiltration cell, (40 p.s.i.), (exclusion limits 10,000 M.W.). The concentrated solution was passed through a Sephadex G-200 column (5.0 cm x 90 cm) which had been equilibrated with 0.05 M phosphate buffer (pH 7.0). The protease was eluted with the same buffer, fractions were collected and the distribution of protein was established by 280 mμ absorbance readings.

Isoelectrofocusing of the Protease:

Low molecular weight ampholines were used of the range pH 3.0 to

6.0 (8%) and pH 3.0 to 5.0 (40%). Both the ampholines and the protease were randomly distributed throughout the column before application of the potential gradient. For detailed instructions refer to LKB 8100 Ampholine Instruction Manual.

Amino Acid Analysis of the Protease:

A protease sample was hydrolyzed under vacuum with 6 N HCl for 18 hr at 105 C. Approximately 0.3 mg of protein hydrolysate was analyzed using the Beckman amino acid analyzer.

Effect of Temperature on Protease Activity:

Protease activity at various temperatures was determined using 2.0% casein as the substrate. Samples of 2.0% casein in 0.1 M phosphate buffer (pH 6.7) were held at temperatures ranging from 30 C to 75 C in a thermal gradient apparatus. The enzyme preparation was diluted 1/50 in 0.05 M phosphate buffer (pH 7.0) and 0.2 ml samples added to the preheated casein. The reactions were incubated for 45 min.

Gel Disc Electrophoresis:

Gel disc electrophoresis was carried out in a standard gel (7%) which was stacked at pH 8.9 and run at pH 9.5 according to the method outlined in the Canalco Model 6 system instruction booklet, Canal Industrial Co.,

Bethesda, Md. The enzyme samples were run in duplicate. After electrophoresis one gel was immediately stained for protein with aniline black, while the duplicate gel was used to assay for various activities. Protease activity and amylase activity were measured by laying the gels on a flat surface on an agar medium containing an appropriate substrate. The enzymes could then diffuse from the bands in the gel into the agar layer.

Protease Activity at: pH 4.0 - An underlay of 0.8 M acetate buffer (pH 35) with 2.0% agar was made in a petri dish. 1.0% casein plus 1.0% agar were mixed and added as an overlay.

pH 6.0 - Glass slides were layered with 1.0% casein made up in 0.2 M phosphate buffer (pH 6.0) plus 1.0% agar.

pH 11.0- Glass slides were layered with 1.0 casein plus 1.0% agar made up in 0.8 M sodium carbonate.

Amylase Activity: Glass slides were layered with 1.0% starch plus 1.0% agar made up with 0.2 M phosphate buffer at pH 6.0 only.

The duplicate gels were placed on the various slides and plate at 37 C for 30 min. The gels were then removed and a 12% $HgCl_2$, 16.5% HCl solution was applied to the casein slides and plate. The starch

slides were flooded with Lugol's iodine. The results of the casein and starch slides were compared to the protein stained acrylamide gels.

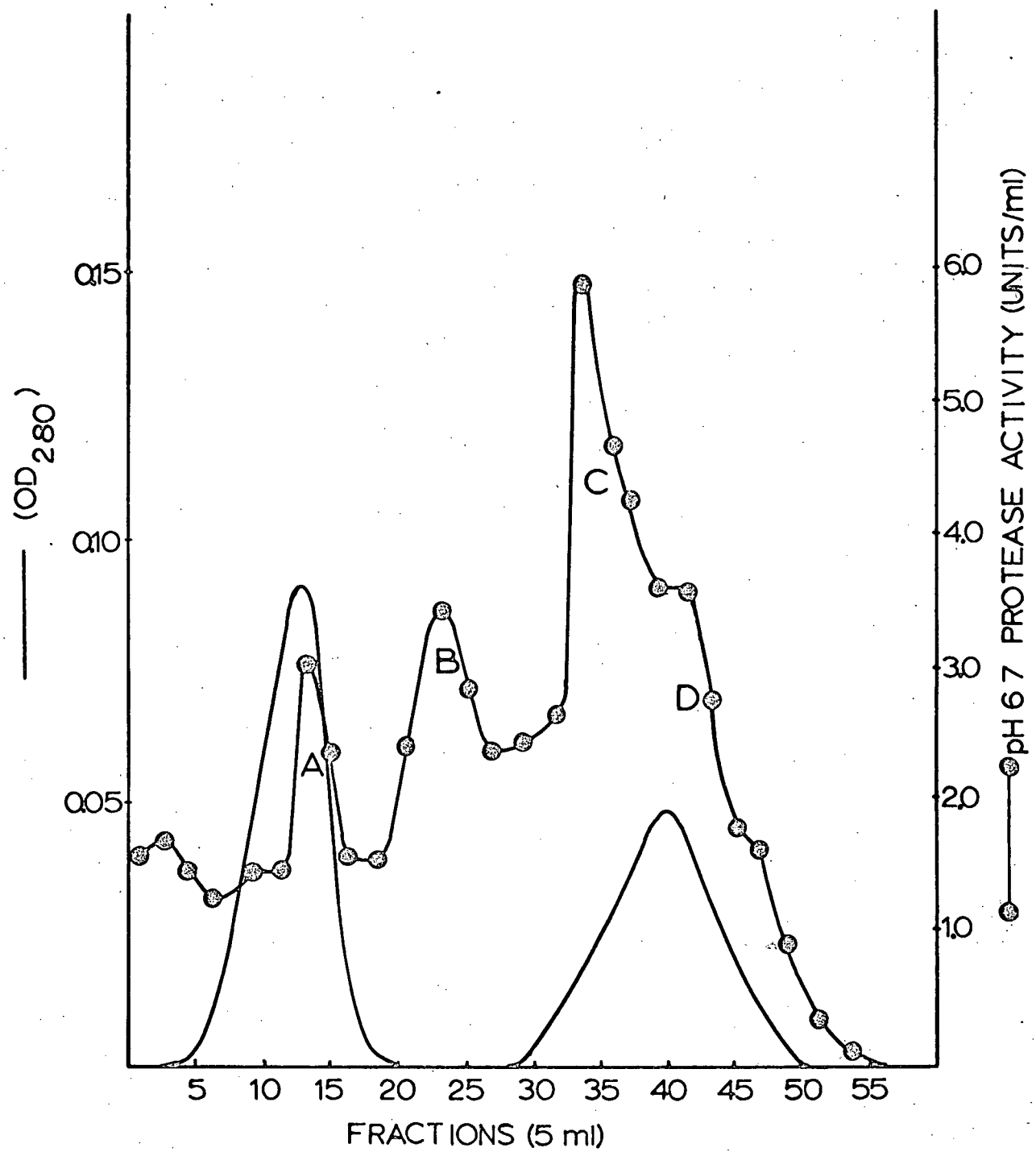
RESULTS AND DISCUSSION

Estimation of Molecular Weight of Crude Protease

The cell-free protease from a 48 hr culture supernatant of B. amylophilus H-18 was precipitated by saturating the solution with ammonium sulfate. The solution was allowed to stand in the cold (4 C) for 12 hr and the precipitate was collected by centrifuging at 8700 xg for 40 min. The precipitate was dissolved in 0.05 M phosphate buffer, pH 7.0 and brought up to a volume of 3.0 ml. Figure 1 shows the elution pattern obtained upon filtration of the redissolved precipitate through a Sephadex G-200 column (2.5 cm x 45 cm) which was equipped with an upward flow adaptor. The column was equilibrated and the protease eluted with 0.05 M phosphate buffer, pH 7.0.

The proteolytic activity appeared in four peaks. Peak A was in the void volume region and the molecular weight was estimated to be greater than 1,000,000 (Andrews, 1961). The protease in peak B had a molecular weight of approximately 130,000 and was of high specific activity. Peaks C and D had higher protein concentrations but the molecular weight of the protease was less than 100,000, suggesting that the protease was no longer covalently attached to membrane fragments as in the situation found by Blackburn (1968 b).

Figure 1. Gel filtration of ammonium sulphate precipitated crude enzyme through Sephadex G-200 (2.5 cm x 45 cm). Eluent: 0.05 M phosphate buffer, pH 7.0. Sample load: 6 mg of protein in 3.0 ml
†, Blue dextran from a separate run.


0.15
0.10
0.05
(OD280)
6.0
5.0
4.0
3.0
2.0
1.0
pH 6 7 PROTEASE ACTIVITY (UNITS/ml)
A
B
C
D
5
10
15
20
25
30
35
40
45
50
55
FRACTIONS (5 ml)

Properties of the Separated Fractions from Gel Filtration:

Table I summarizes the properties of protease activity at various pH values of the peaks A to D obtained from gel filtration using Sephadex G-200. The ratios $E_{280}:E_{260}$ indicated that the amounts of nucleic acid present in peaks A, B, C and D were 3.6%, 3.8%, 5.0% and 4.2% respectively (Layne, 1957). This was a lower nucleic acid content compared to the 6.0% nucleic acid found by Blackburn (1968 b), suggesting that little cell lysis had taken place.

If only a single protease was present, the ratio of activities at the various pH values in each fraction should have been the same. Although the activity was greatest at pH 6.7 in all four fractions, the ratios of activities at various pH values did differ from fraction to fraction. However, enzyme complexes may not have the same pH profile as an enzyme free in solution.

Peak B showed a purification as high as eight times in the case of the pH 6.7 value. It therefore appeared that Sephadex G-200 gel filtration would be a useful step in the purification of protease from B. amylophilus H-18.

Time Course of Protease Liberation:

Preliminary experiments showed that proteolytic enzymes, active over a wide pH range, were liberated into the culture supernatant.

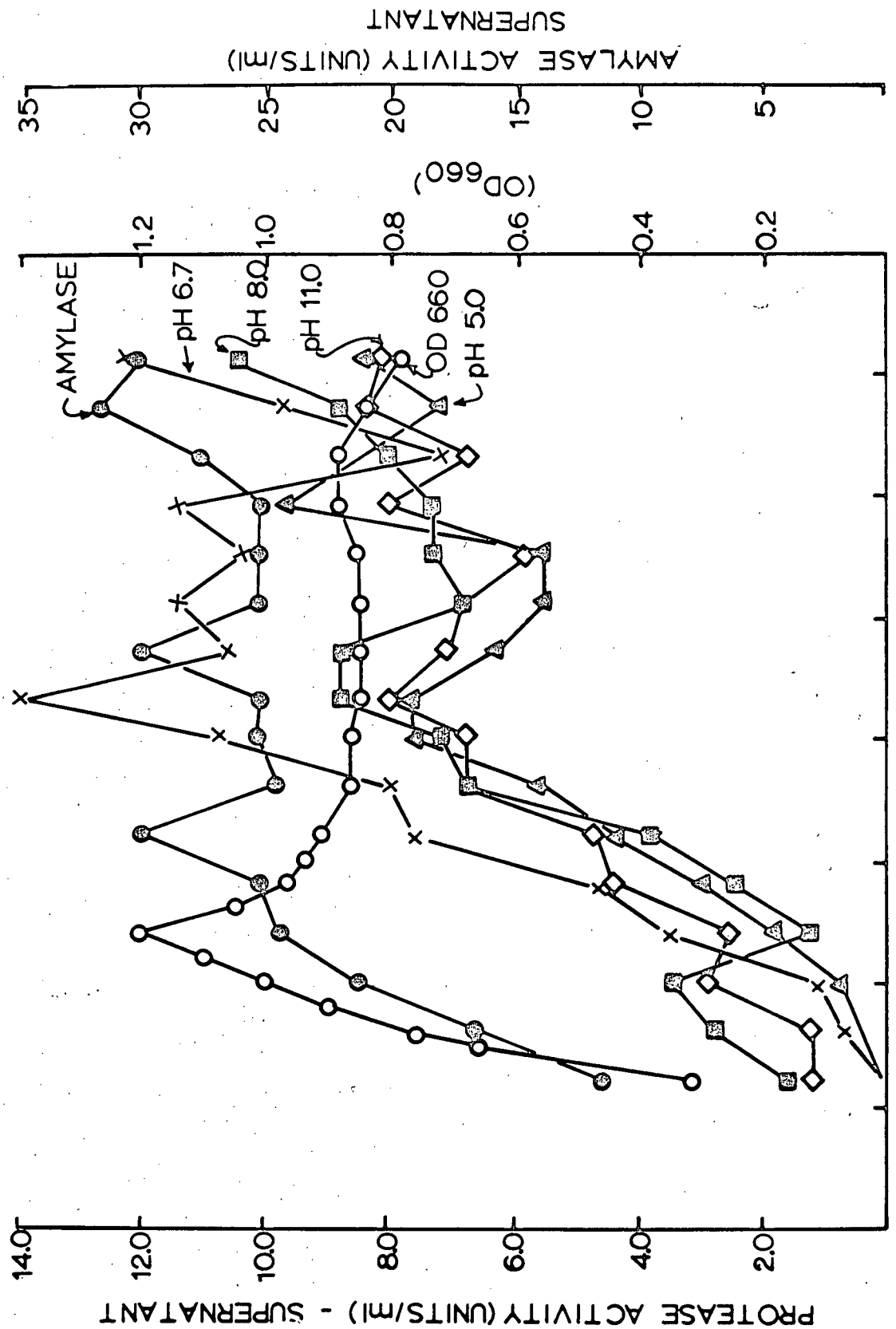
Fraction	Total Volume	$E_{280}:E_{260}$	Protein (µg/ml)	Protease Activity Units/ml (Ratio)				Specific Activity				Purification				Mol. Wt.
				pH				pH				pH				
				5.0	6.7	8.0	11.0	5.0	6.7	8.0	11.0	5.0	6.7	8.0	11.0	
A.	20 ml	0.965	2.88	0.38 (1.8)	2.12 (10)	- -	0.10 (0.47)	132	736	-	347	1.13	6.5	-	3.37	10^6
B.	25 ml	0.945	2.88	0.65 (2.5)	2.60 (10)	0.54 (2.0)	0.71 (2.7)	226	900	187	247	1.92	8.0	3.2	2.40	130,000
C.	30 ml	0.875	28.8	2.36 (1.15)	4.57 (10)	1.47 (0.9)	0.98 (1.1)	82	162	51	34	-	1.4	-	-	20,000
D.	35 ml	0.900	31.4	0.32	2.83	0.27	0.32	10	9.3	8.6	10	-	-	-	-	5,000
Dissolved $(NH_4)_2SO_4$ Precipitate	3.0 ml	0.915	2000	235	226	117	206	117	113	58	103					

Table I. Properties at various pH values of fractions from Sephadex G-200 gel filtration and from dissolved ammonium sulfate precipitate.

Since it seemed probable that no single enzyme could be responsible, the time course of the liberation of protease activity at pH 5.0, 6.7, 8.0 and 11.0 was examined in the hope that the pH 6.7 activity might be liberated at a different time from the other activities. The characteristic sharp fall in cell density once the maximum was reached, did not coincide with a liberation of protease activity (Fig. 2). However, on prolonged incubation, the proportion of cell-free protease increased and this and the sharp decrease in culture density at the end of logarithmic growth is characteristic of many rumen bacteria (Bryant and Robinson, 1961). This substantiated the data (Fig. 1) that the cell-free protease was not attached to disintegrated cell particles. Protease, active at all the pH values examined, was liberated progressively throughout the logarithmic and stationary phases, reaching a maximum 12 hr after the culture had reached maximum cell density (Fig. 2). Fluctuations in protease activity occurred but these fluctuations were coincident for protease activity over the pH range, which suggested that one enzyme might be responsible for all the activity which was observed.

The time course of amylase liberation was also followed and was seen to reach a peak activity much earlier, only 5 hr after maximum cell density (Fig. 2). This activity also fluctuated subsequently, but not in the same pattern as the protease activity.

Figure 2. Cell density (0 - 0), protease activity at pH 5.0 (Δ - Δ), 6.7 (X - X), 8.0 (■ - ■), 11.0 (□ - □) and amylase (● - ●) in the culture supernatant are plotted against hours of incubation.


PROTEASE ACTIVITY (UNITS/ml) - SUPERNATANT
14.0
12.0
10.0
8.0
6.0
4.0
2.0
AMYLASE ACTIVITY (UNITS/ml)
SUPERNATANT
35
30
25
20
15
10
5
(OD660)
1.2
1.0
0.8
0.6
0.4
0.2
AMYLASE
pH 6.7
pH 8.0
pH 11.0
OD 660
pH 5.0
5
10
15
20
25
30
35
AGE OF CULTURE (HR)

Protease Purification:

The procedure is described in the Methods and the results are summarized in Table II.

Growth:

A good yield of protease was obtained in the 23 hr culture supernatant. This represented 80% of the total protease (cell-bound and cell-free) which had reached a maximum at 13 hr. A sample of the supernatant was dialyzed thoroughly against 0.05 M phosphate buffer, pH 7.0, to remove the cysteine and tryptose peptides so that the protein content could be determined accurately.

Concentration:

The concentration step was successfully accomplished by binding the protease to DEAE-Sephadex. The eluted, dialyzed protease represented 75% of the starting material. Although this step gave only a 4-fold purification, it did reduce the volume of enzyme solution from 29 to 2.6 litres.

DEAE-Chromatography:

Further purification (3-fold) and concentration (2,600 ml to

Table II. Purification of *B. amylophilus* H-18 protease. Recoveries are calculated as if all material was used.

	Sample	pH of assay	Volume	Units /ml	Total Units	Protein (mg/ml)	Sp. Act.	X Pur	% recov.
1.	23 hr sup.	5.0	29 l	7.2	209,000	0.317	22.7	1	100
		6.7	29 l	9.4	270,000	0.317	29.7	1	100
		8.0	29 l	8.0	232,000	0.317	25.4	1	100
		11.0	29 l	8.5	246,000	0.317	27.0	1	100
2.	DEAE elution	6.7	2600 ml	78.0	202,000	0.618	126	4	75
3.	DEAE column #57-84 Fig.3	6.7	500 ml	270	135,000	0.725	373	126	50
4.	DEAE column #85-89 Fig.3	6.7	90 ml	62	5,580	1.010	61.5	2.8	2.0
5.	#3 conc.	5.0	71 ml	4800	340,000	4.0	1200	53.3	116
		6.7	71 ml	4670	330,000	4.0	1165	40.7	126
		8.0	71 ml	3260	228,000	4.0	815	32.1	84
		11.0	71 ml	4300	308,000	4.0	1070	39.6	125
6.	40 ml #5 through Sephadex G-200 #69-87 Fig.4	6.7	230 ml	420	96,500	0.056	7440	251	62.6
7.	31 ml #5 through Sephadex G-200								
	A. #71-81	6.7	138 ml	284	39,200	0.056	5040	170	31.6
	B. #83-93	6.7	138 ml	112	15,300	0.058	1930	65	12.3
8.	#6 conc.	5.0	35.5 ml	3700	131,000	0.330	11,200	494	107.0
		6.7	35.5 ml	3900	138,000	0.330	11,900	404	89.1
		8.0	35.5 ml	2770	98,000	0.330	8,400	286	83.0
		11.0	35.5 ml	3800	135,000	0.330	11,500	425	108.0
9.	#7 conc A.	5.0	14.0 ml	5600	78,500	0.340	16,500	725	85.0
		6.7	14.0 ml	5370	75,000	0.340	16,100	545	62.7
		8.0	14.0 ml	4600	64,500	0.340	13,500	455	85.0
		11.0	14.0 ml	5350	75,300	0.340	15,700	580	69.4
	#7 conc. B.	5.0	11.5 ml	2600	30,000	0.330	7,900	348	32.1
		6.7	11.5 ml	2580	30,800	0.330	7,800	265	24.8
		8.0	11.5 ml	2150	24,800	0.330	6,500	256	24.1
		11.0	11.5 ml	2500	28,800	0.330	7,600	281	26.6
10.	Electrofocusing								
	Peak I	6.7	1.9 ml	3760	7,140	0.19	37,600	1265	27.7
	Peak II	6.7	0.9 ml	704	633	0.87	7,270	245	2.7

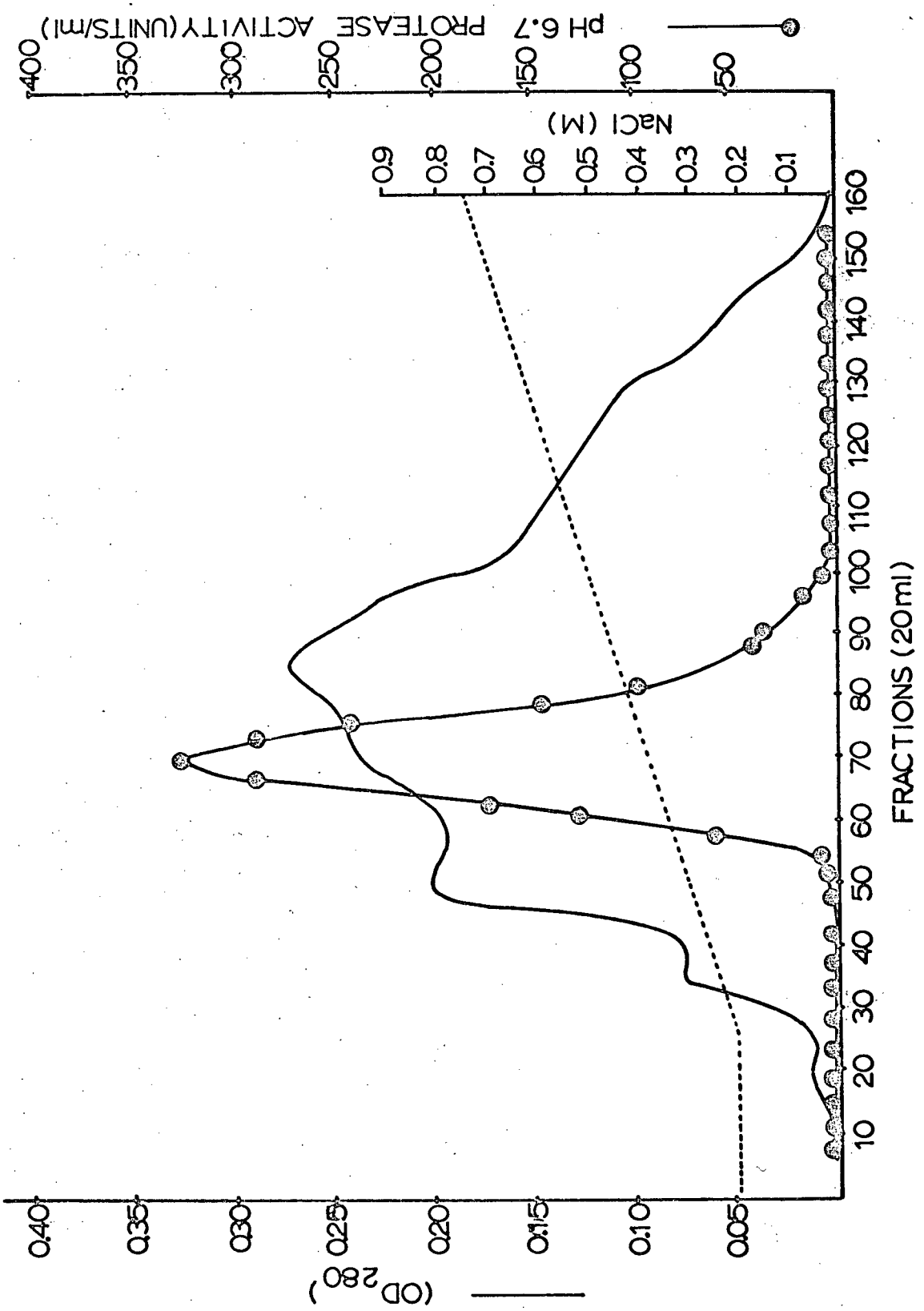
500 ml) were achieved by adsorption on a DEAE-Sephadex column with subsequent elution by a sodium chloride gradient (Fig. 3). In this step, 67% of the protease activity was recovered in a single peak (Table II, #3), with only 3% of low specific activity protease in the trailing shoulder of this peak.

Concentration of the pooled protease led to an apparent increase in activity (244%) and a decrease in total protein, resulting in a 3.0-fold purification. At this point the protease activities at pH 6.7 and pH 8.0 had both been purified 40-fold overall. Neither had the protease activities at pH 5.0 and 11.0 been separated from the pH 6.7 protease. It appeared, therefore, that a single enzyme was responsible for the protease activities over the range pH 5.0 to 11.0. A point of considerable interest was the failure of the purification steps employed to this point to separate the amylase from the protease.

G-200 Sephadex:

Preliminary experiments (Table I), indicated gel filtration to be a useful method in resolving the protease activity at different pH values which were used in the assays. Some of the concentrated enzyme solution obtained after the DEAE-Sephadex step was passed through a G-200 Sephadex column (Table II, #6). Protease activity was eluted in a single peak, termed peak A,

Figure 3. Chromatography of B. amylophilus H-18 protease on DEAE Sephadex A-50.
Eluent: 0.05 M phosphate buffer, pH 7.0; a linear gradient of NaCl (0.2 to 0.75 M) was applied for the elution of protein.

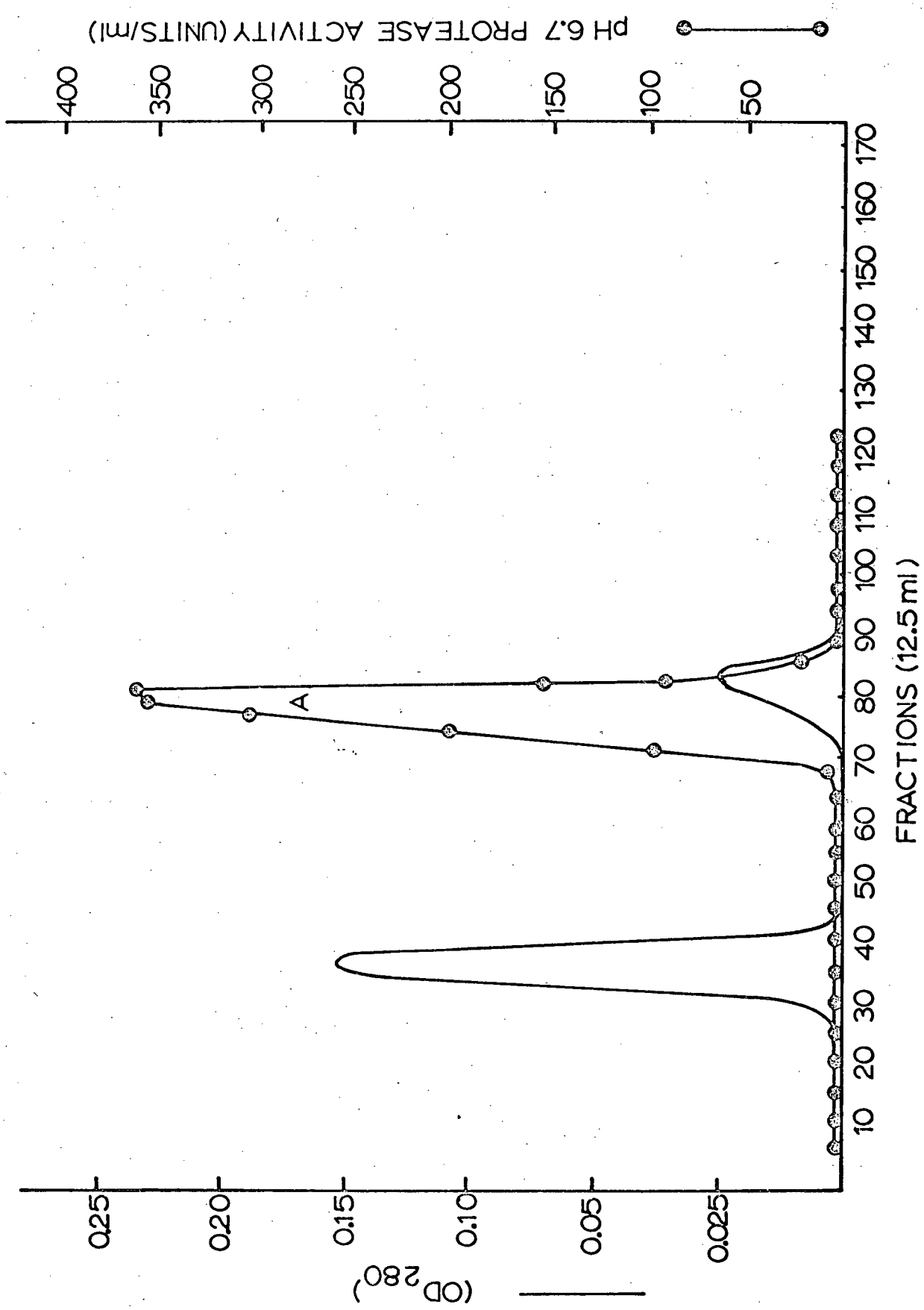

(OD280)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160
FRACTIONS (20ml)
NaCl (M)
0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1
pH 6.7 PROTEASE ACTIVITY (UNITS/ml)
400 350 300 250 200 150 100 50

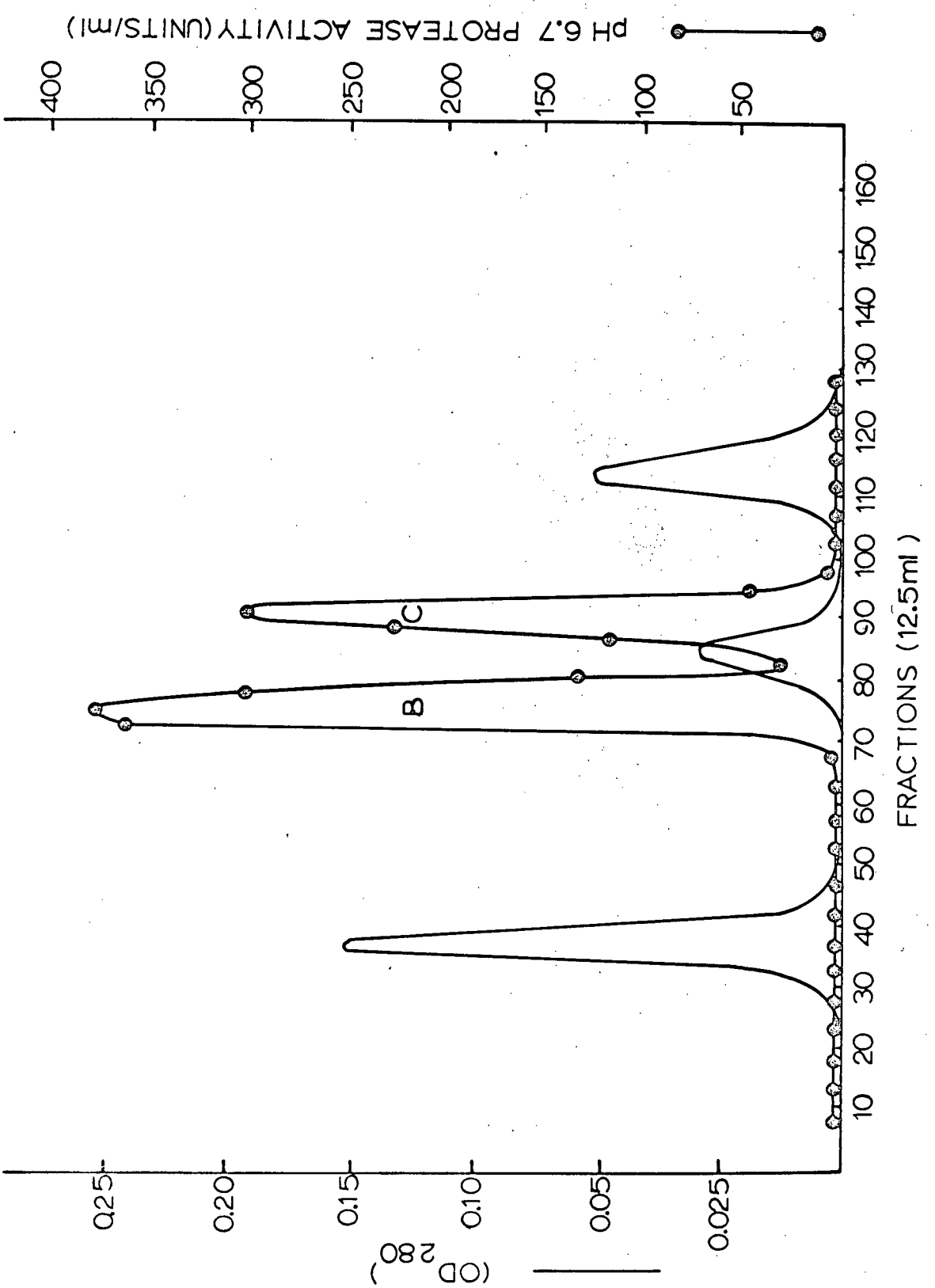
with a 36.7% recovery and a 6.4-fold purification. When the pooled fractions were concentrated again, there was an apparent increase in proteolytic activity, resulting in a 143% recovery and a 1.6-fold increase in activity (Table II, #8). The protease was calculated (Andrews, 1964) to have a molecular weight in the 60,000 range (Fig. 4). The Sephadex G-200 column was repacked to improve its flow characteristics. When the remainder of the concentrated enzyme solution obtained after the DEAE Sephadex step was passed through this column, the protease activity was eluted in two peaks (Fig. 5).

The enzyme in one peak (Fig. 5, B) had a molecular weight of approximately 60,000. The enzyme in the second peak (Fig. 5, C) had a molecular weight of approximately 27,000. Of the enzyme applied to the column, 27% appeared in peak B, with a 4.3-fold purification, and 10.4% appeared in peak C with a 1.7-fold purification. When the pooled fractions were concentrated again there was an apparent increase in proteolytic activity: 3.2-fold and 4.0-fold for B and C respectively, with recoveries of 191% and 201% (Table II, #9). The sum of the total activity in peaks A, B and C represented a 90% recovery of the proteolytic activity present in the original 29 litres of culture medium. The possibility was recognized that the protease in peak B might be a dimer of that in peak C. Corroborative evidence for this was found in the fact that both peaks contained protease

Figure. 4. Gel filtration of a portion of sample #5 (Table II) through a Sephadex G-200 column (5.0 cm x 90 cm). Eluent: 0.05 M phosphate buffer, pH 7.0.

Sample (Table II)	Volume	Units/ml	Total Units	Protein (mg/ml)	Sp.Act.	Total Protein (mg/ml)	X Pur	% Recov.
#5 applied	40 ml	4670	263,000	2.25	1165	90.0		
A #69-85 (#6)	230 ml	420	96,500	0.056	7440	13.0	6.4	36.7
A conc. (#8)	35.5 ml	3900	138,000	0.33	11900	11.7	1.6	143.0


pH 6.7 PROTEASE ACTIVITY (UNITS/ml)
400
350
300
250
200
150
100
50
A
(OD 280)
0.25
0.20
0.15
0.10
0.05
0.025
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170
FRACTIONS (12.5 ml)


pH 6.7 PROTEASE ACTIVITY (UNITS/ml)
400
350
300
250
200
150
100
50
(OD 280)
0.25
0.20
0.15
0.10
0.05
0.025
B
C
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160
FRACTIONS (12.5ml)

active in the range pH 5.0 to 11.0 and thus very similar in properties, although different in size.

When gel filtration of peak C (Fig. 5) was repeated using Sephadex G-200, the protease appeared in the 60,000 rather than the 30,000 molecular weight range where it was previously isolated. This reinforced the hypothesis that the 60,000 and 30,000 molecular weight proteases were in equilibrium with the dimeric form predominating.

Ultracentrifugation:

Ultracentrifugation of peak C (Fig. 5) revealed only one homogenous peak (Fig. 6). Since the repeated run of peak C on Sephadex G-200 gave only 60,000 molecular weight protease, it is reasonably safe to assume that the single peak obtained from the ultracentrifugation of peak C is the 60,000 molecular weight protease.

Effect of pH on Protease Activity:

It was considered unusual that protease activity at all pH values from 5.0 to 11.0 should remain together despite the considerable degree of purification. Blackburn (1968 b) showed activity over a wide range and Blackburn's unpublisheed results

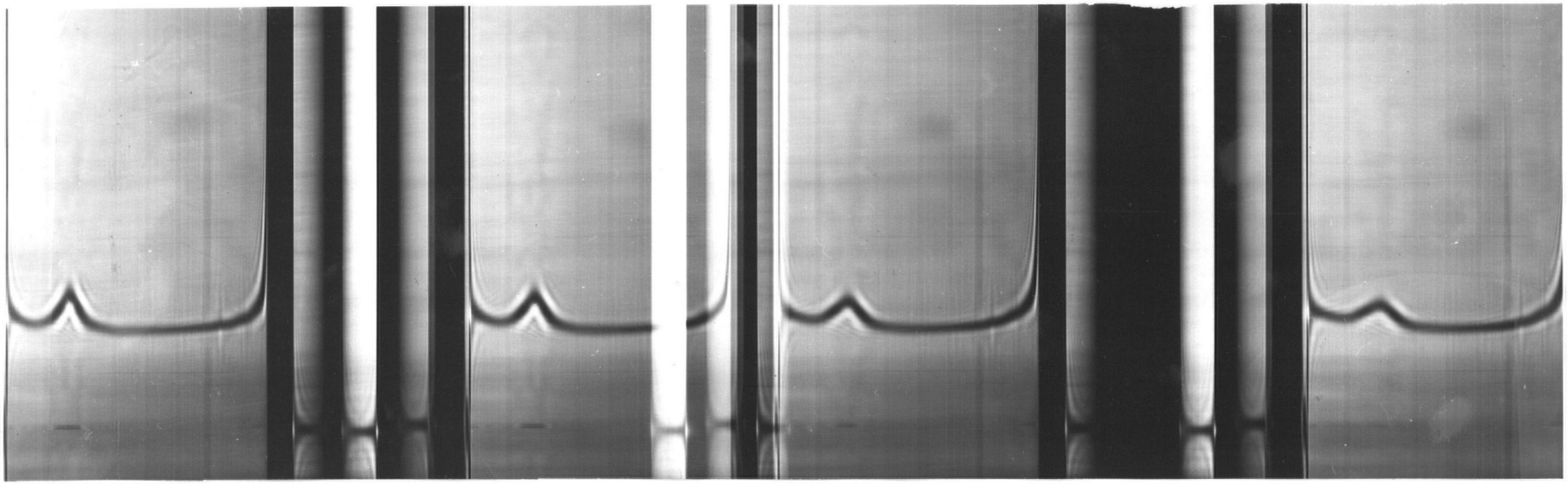


Figure 6. The enzyme sample was run in a synthetic boundary cell at 56,000 rev/min in 0.1 M phosphate buffer (pH 7.0). Pictures were taken 5 min after attaining full speed at 4 min intervals.

indicated proteolytic activity over a pH range from 4.0 to 11.0. It was assumed that the peaks of activity 5.0, 6.7, 8.0 and 11.0 were due to different proteases. However, this may not be the case, since Hofsten et al (1965) characterized a protease from an Arthrobacter which demonstrated proteolytic activity from pH 4.0 to 11.0 and Matsubara et al (1958)crystallized a protease from Bacillus subtilis N which was active from pH 5.5 to 9.5.

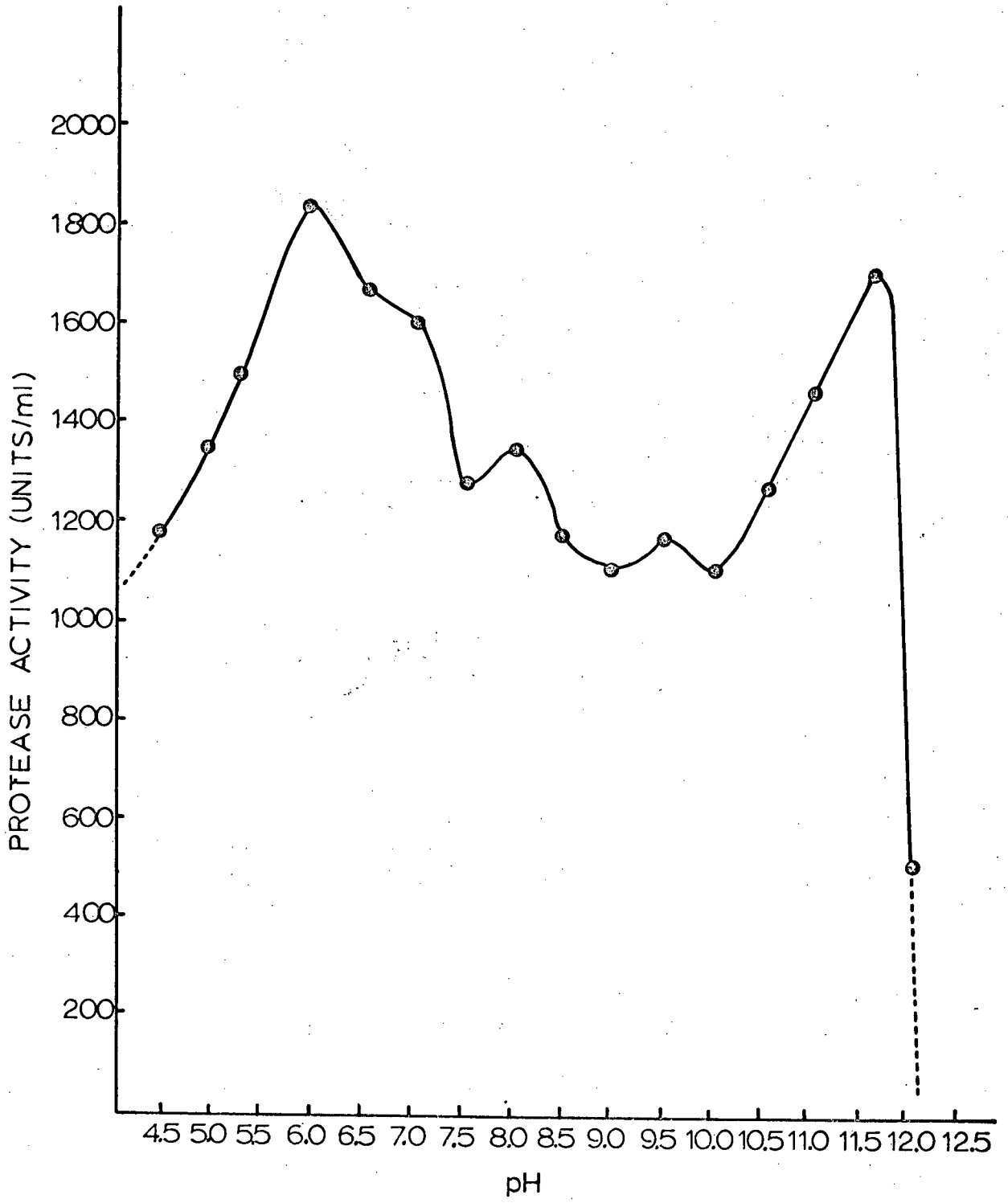
The protease in peak A was active over the pH range from pH 4.5 to 11.5 (Fig. 7). There were major peaks of activity at pH 5.0 and 11.5, with minor peaks at pH 8.0 and possibly 9.5. Thus, unlike the single broad peak of activity obtained by Matsubara et al (1958), there were distinct peaks of activities at either ends of the pH activity range.

Since the curve of protease activity against pH (Fig. 7) for the purified protease showed pH optima differing from those of the starting material, the possibility again had to be considered that more than one protease was present. If so, the fact that the activities had not been separated from each other at this point in the purification indicated that they might be closely related in structure.

Isoelectrofocusing:

Preliminary evidence from DEAE-Sephadex absorption studies

Figure 7. Effect of pH on activity of purified protease (Table II, #8). Reaction mixture contained, (0.2 ml), enzyme and (1.8 ml), 2% casein (pH 4.5 to 12.0).

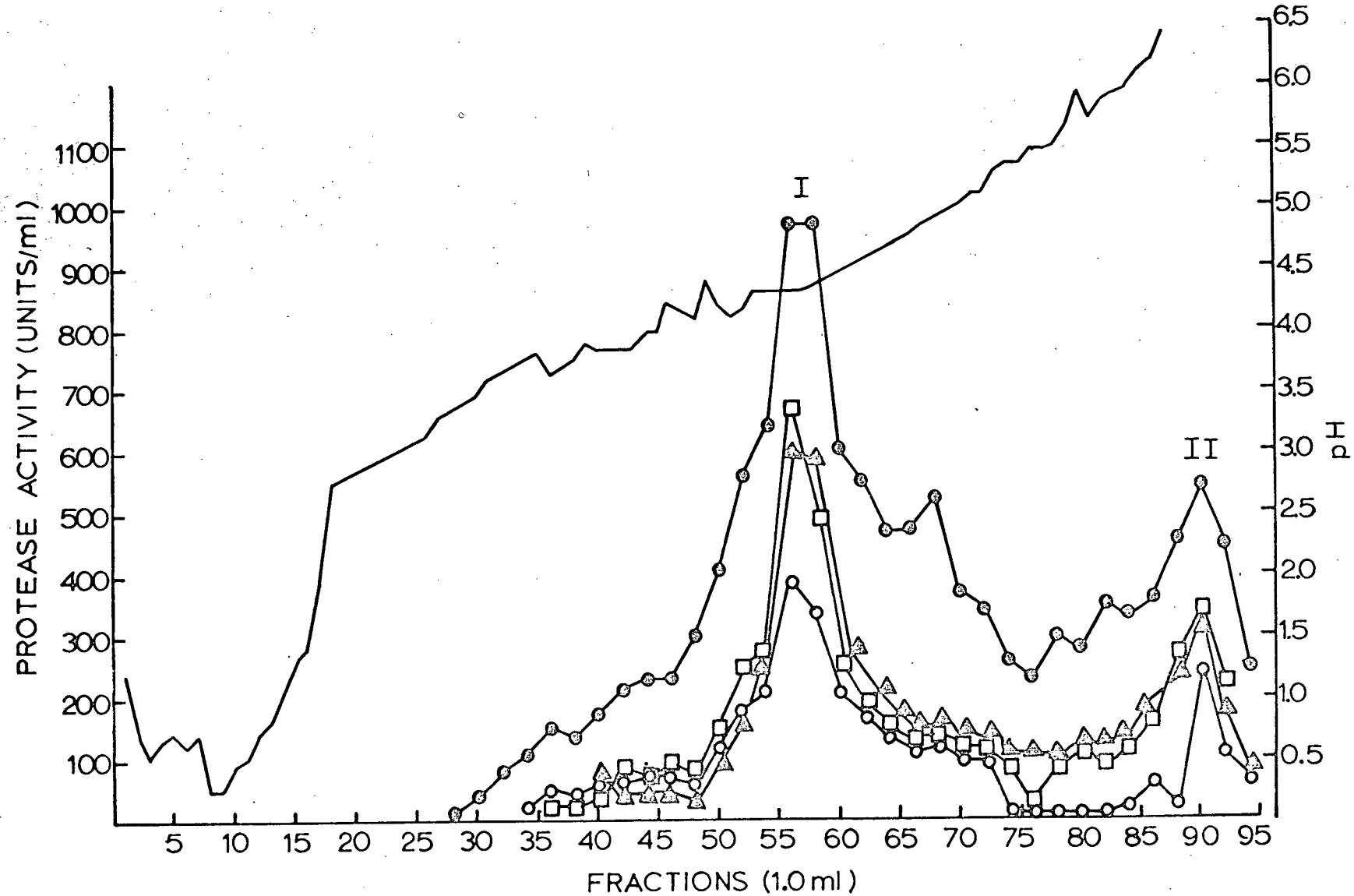

PROTEASE ACTIVITY (UNITS/ml)
2000
1800
1600
1400
1200
1000
800
600
400
200
4.5 5.0 5.5 6.0 6.5 7.0 7.5 8.0 8.5 9.0 9.5 10.0 10.5 11.0 11.5 12.0 12.5
pH

suggested that the protease was an acidic protein with an isoelectric point below pH 5.0. Attempts to bind the protease to the cation exchanger, SE-Sephadex, C-50, at pH 4.2 resulted in loss of protease activity, although the protease itself was stable at low pH values. Therefore, Isoelectrofucusing was used to purify the protease further and to separte the proteases active at different pH values.

The starting material in the electrofocusing experiment was protease which appeared in the 60,000 molecular weight range after gel filtration with Sephadex G-200 (Table II, #8). Proteolytic activity in the fractions obtained from the electrofocusing experiment appeared in two major regions (Fig. 8). The proteolytic activity at pH 5.0, 6.7, 8.0 and 11.0 all gave the same profile in the two major peaks. The enzyme in peak I had an isoelectric point at pH 4.3, that in peak II an isoelectric point at pH 7.95. Disc gel electrophoresis of concentrated peak I enzyme showed the presence of proteases but no amylase, even though the starting material contained amaylase. The fact that only one band of active protease was seen after disc gel electrophoresis could be accounted for as the length of gel used was too short to obtain the resolution required to observe two proteolytic components. Therefore it was still possible that the dimeric and monomeric forms existed in peak I. Disc gel electrophoresis of peak II enzyme revealed very many proteins. It was likely that

Figure 8. Protease profile from an electrofocusing experiment with a superimposed pH curve (pH 3.0 to 6.0). Symbols: Protease activity at pH 5.0 (0 - 0), 6.7 (0 - 0), 8.0 (Δ - Δ) and 11.0 (-).

Sample (Table II)	Volume	Units/ml	Total Units	Protein Total (mg/ml)	Sp.Act.	X Pur	% Recov.
#8 applied	5.8 ml	3900	22,700	1.91	11,900		
Peak I (#53-63)	1.9 ml	3760	7,140	0.19	37,600	3.2	31
Peak II (#84-92)	0.9 ml	704	633	0.87	7,270	0.6	3


PROTEASE ACTIVITY (UNITS/ml)
1100
1000
900
800
700
600
500
400
300
200
100
I
II
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
pH
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95
FRACTIONS (1.0 ml)

peak II contained degradation products of protease molecules, some of which still retained their proteolytic activity.

The contents of the tubes in each peak were pooled, dialyzed to remove ampholines and then concentrated. The protein contents of these concentrated solutions were very low and could not be determined by the Lowry Method without using all the material. Consequently, the protein contents were calculated from the recovery of $E_{280\ m\mu}$ absorbing material and the known protein contents of the sample run in the electrofocusing apparatus (Table II, #8). For the solution peak I there was a 3.2-fold purification with a 32% recovery of pH 6.7 protease. In this experiment the potential gradient was applied for 48 hr, after which time there was visible precipitation of protein within the electrofocusing column. The experiment was repeated using a narrow range of ampholites (pH 3.0 to 5.0) and a 24 hr gradient. In this case, the isoelectric point of the enzyme in peak I was at pH 4.25. However, application of the potential gradient for 24 hr was insufficient time to concentrate the protease and so the peak of activity was more diffuse than in the 48 hr run.

The purified protease (Table II, #8) had now been separated into two components by both gel filtration and by electrofocusing. However, since the protease pH activity profiles were identical after each of these steps, it was still possible that there was only a single protease or perhaps a family of very closely related

proteases, as was previously suggested.

Acrylamide Gel Electrophoresis:

Acrylamide gel electrophoresis (Fig. 9) of the purified protease preparation (Table II, #8) tended to confirm the data from the gel filtration: that two proteases were present which were presumably being separated on the basis of size or charge. Therefore, it was anticipated that samples #9 A and #9 B (Table II) containing proteases with molecular weights of 60,000 and 30,000 respectively, would give different gel electrophoretic patterns. In fact they both gave the same protease pattern as that shown in Fig. 9, the only difference being the absence of three amylase bands from the 30,000 molecular weight fraction. Each of the protease bands showed proteolytic activity from pH 4.0 to 11.5. Thus, there was no qualitative difference between their activities; quantitatively protease band #1 contained more protein and more proteolytic enzyme activity. A possible explanation for these results is that the protease is an equilibrium mixture of a monomeric and dimeric form, the dimeric form (protease band #1, Fig. 9) predominating. Disc gel electrophoresis of a sample from electrofocusing peak I also showed the protease protein band #1 but the amylase bands were missing. Although, it was clear that amylase activity could be separated from protease activity, the

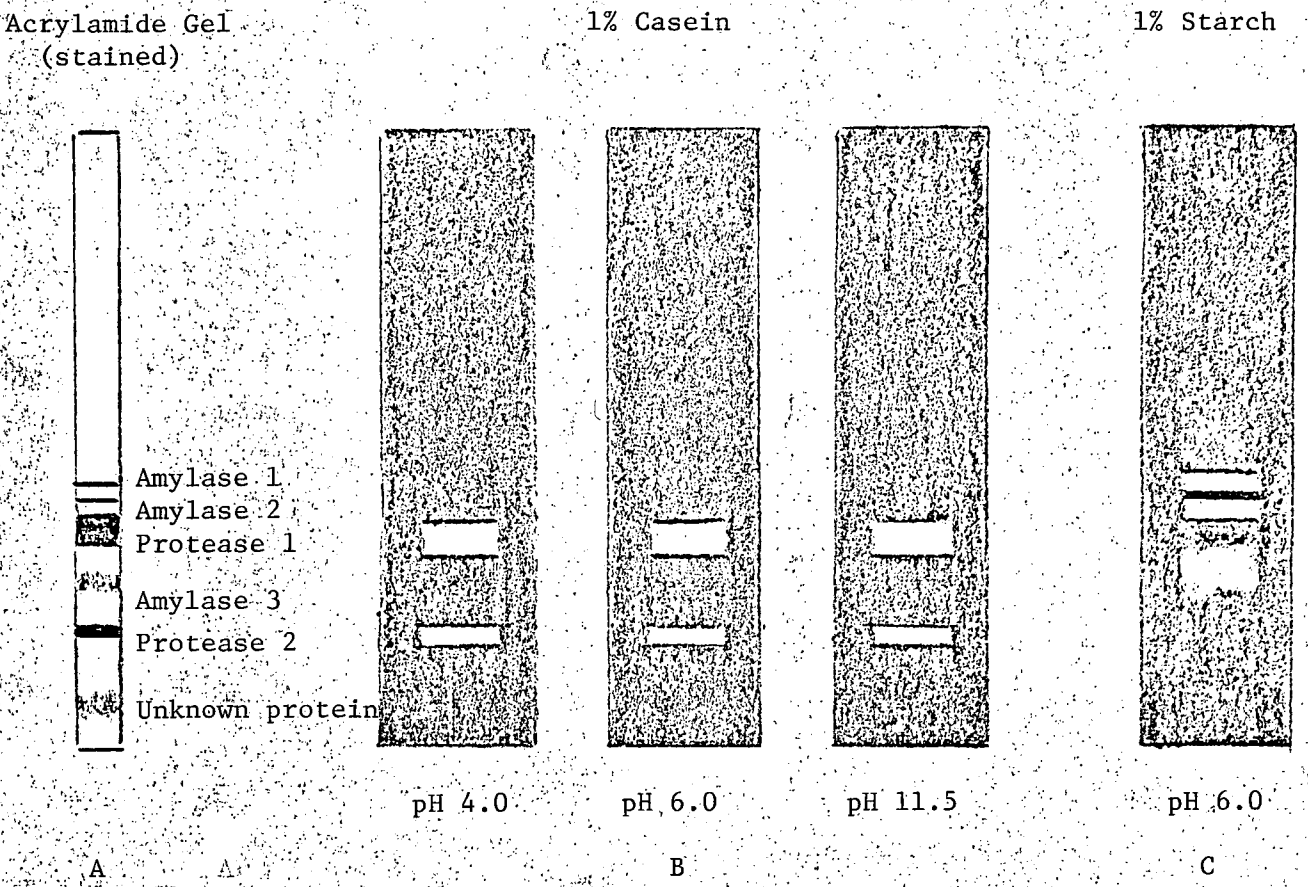


Figure 9. Representation of A, acrylamide electrophoresis gel with stained protein bands. B, casein slides at various pH values after incubation at 37 C for 30 min with acrylamide gels. C, is of a starch slide pH 6.0, after similar treatment. The unhydrolyzed casein was precipitated with $HgCl_2$ and the unhydrolyzed starch was complexed with Lugol's iodine.

fact that it was unresolved by gel filtration from the protease while the latter was purified 404-fold (Table II, #6) indicated that the two types of enzymes were very similar structurally.

Protease Characterization:

Effect of Chemical Reagents on the Protease:

If the protease in sample #8 (Table II) was a dimer, it should have been possible to dissociate it chemically into the 30,000 molecular weight form protease, since sample #5 (Table II) was separable into two components of differing molecular weight by means of gel filtration.

The low concentration of protein in sample #8 (Table II) precluded the use of the Lowry Method for the detection of protease protein in the fractions obtained from gel filtration. Therefore, the chemical techniques used in an attempt to dissociate the dimeric form of the protease would have to be such that the proteolytic activity of the enzyme was not destroyed during prolonged exposure to the chemicals.

To this end, 10^{-3} M EDTA and 4 M urea were used as agents to break bonds which may have been binding the dimeric form of the proteases together (Figures 10 and 11). In both cases, the protease appeared only in the 60,000 molecular weight region after gel

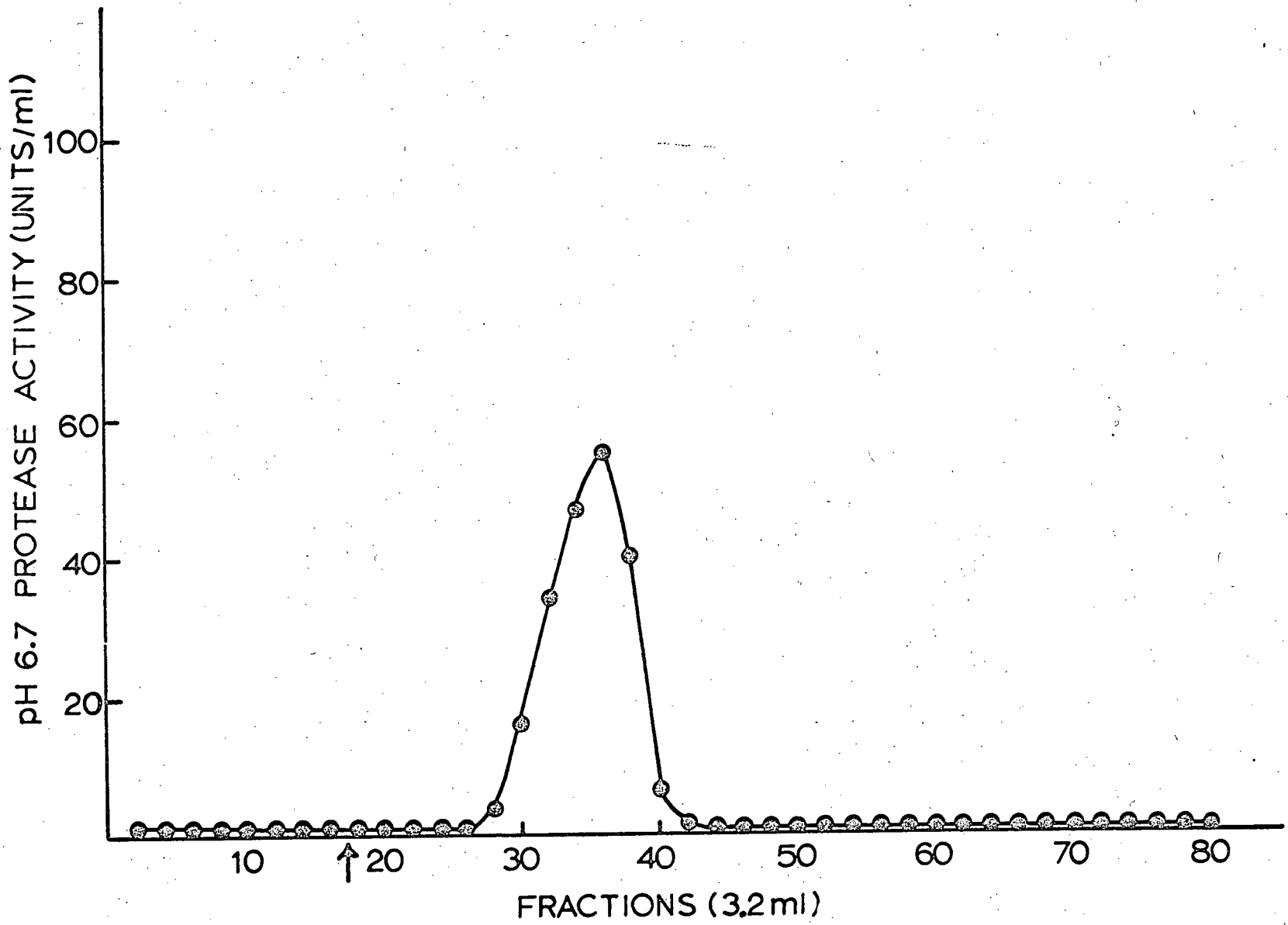
V_o = 54.5ml

V_e = 115.2 ml

V_e/V_o = 2.12

Molecular Weight = 60,000

Figure 10. Gel filtration of(Table II, #8) which had been pre-treated with 10^{-3} M EDTA. Assay mixture contained 0.2 ml of each fraction and 1.8 ml, 2.0% casein, pH 6.7. ↑ Blue dextran.


pH 6.7 PROTEASE ACTIVITY (UNITS/ml)
100
80
60
40
20
10
20
30
40
50
60
70
80
FRACTIONS (3.2 ml)

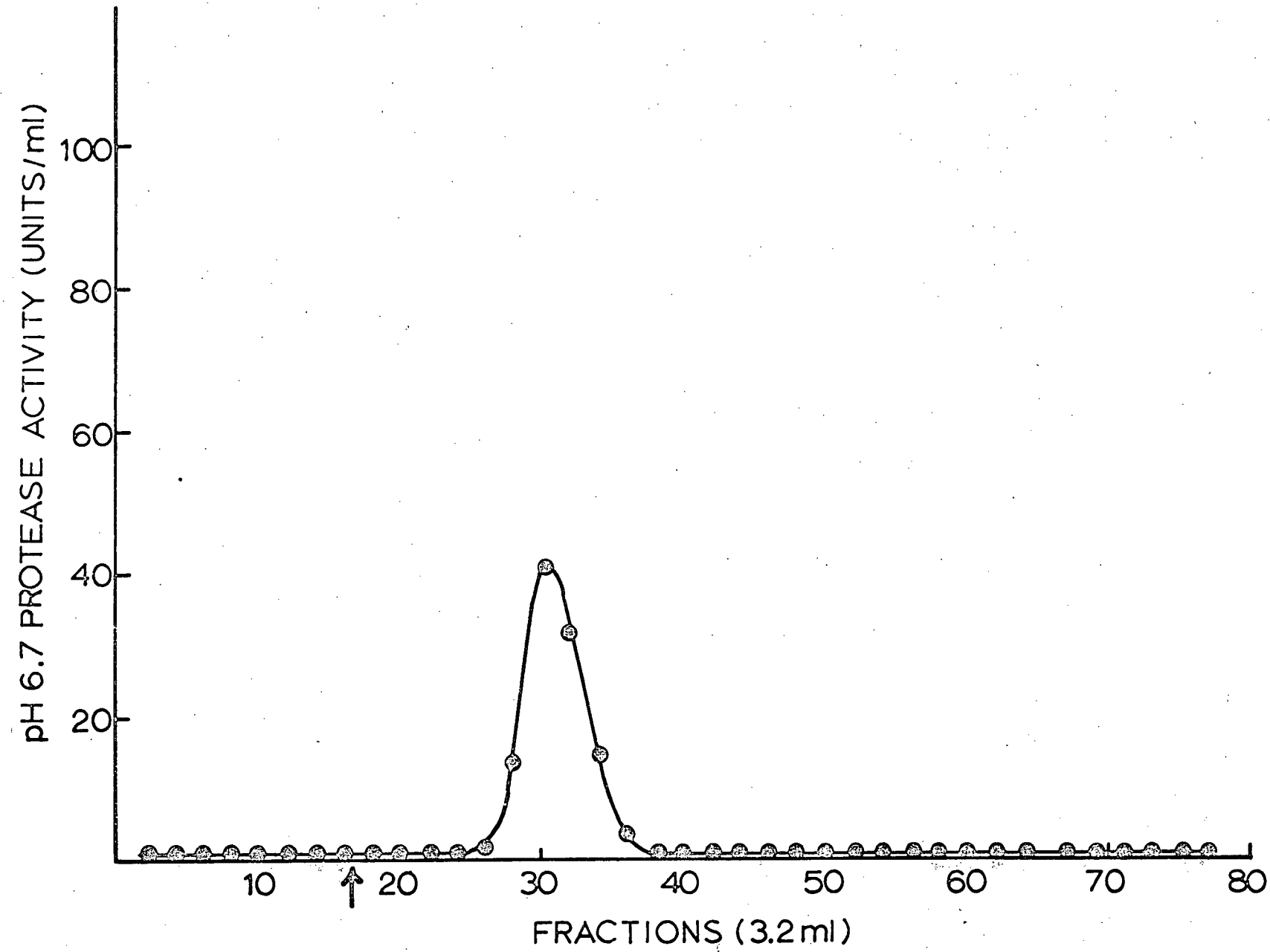
V_o = 51.2 ml

V_e = 99.2 ml

V_e/V_o = 1.94

Molecular Weight = 60,000

Figure 11. Gel filtration of (Table II, #8) which had been pre-treated with 4 M urea. Assay mixture contained 0.2 ml of each fraction and 1.8 ml, 2.0% casein, pH 6.7. ↑ Blue dextran.


pH 6.7 PROTEASE ACTIVITY (UNITS/ml)
100
80
60
40
20
10
20
30
40
50
60
70
80
FRACTIONS (3.2 ml)

filtration with Sephadex G-200. Treatment of sample #8 (Table II) with sodium dodecyl sulphate 10^{-5} M concentration, completely inactivated the protease making it impossible to locate after gel filtration.

Amino Acid Analysis:

Amino acid analysis of the protease (Table II, #8) from B. amylophilus H-18 revealed a complete absence of cysteine, so that disulfide bridges could not be involved in the maintenance of tertiary structure and enzymic activity of the protease molecule. The amino acid composition reported in Table III is compatible with the molecular weight obtained using Sephadex G-200. That is, 27,000 and 60,000 molecular weight protease. That no sulfhydryl groups were present was confirmed by the failure of p-chloromercuribenzoate to inactivate or even react with the enzyme. Hofsten et al (1965) reported half-cysteine to be absent from the oxidized form of protease purified from an Arthrobacter Sp. Pollock (1962) demonstrated a low cysteine content to be a common feature of extracellular bacterial proteins, including a variety of proteases and extracellular enzymes.

Table III. Amino acid composition of the B. amylophilus H-18 protease (Table II, #8).
0.3 mg of protease protein was analyzed.
The molar ratio was adjusted to give a molecular weight of 30,000 by having methionine represent 3 residues.

Amino Acid Residue	µmoles	µgram	Molar Ratio	Molecular Weight of Amino Acids x Number of Residues
Lysine	0.24428	35.70	20	2923
Histidine	0.07301	11.33	6	886
Arginine	0.17281	30.10	14	2488
Aspartic Acid	0.34727	44.22	30	3802
Threonine	0.18901	22.53	15	1702
Serine	0.13958	14.67	11	1201
Glutamic Acid	0.33579	61.65	27	4983
Proline	0.24909	28.67	20	2302
Glycine	0.32026	24.04	26	1931
Alanine	0.20847	18.15	17	1527
Half-cystine	-	-	-	-
Valine	0.17615	20.64	14	1673
Methionine	0.03546	5.30	3	426
Isoleucine	0.09903	13.00	8	1124
Leucine	0.12732	16.70	10	1349
Tyrosine	0.04940	9.00	4	719
Phenylalanine	0.07219	12.00	4	944
Total		367µg		29,980

Effect of Temperature on Protease Activity:

The optimum temperature for activity of the purified protease (Table II, #8), was between 60 C and 65 C (Fig. 12). This is similar to the proteases of Pseudomonas aeruginosa (Morihara, 1963) and Mucor pusillus (Somkuti and Babel, 1968). An Arrhenius plot showed a logarithmic increase in enzyme activity from 30 C to 50 C (Fig. 13). Above 50 C there was an irreversible inactivation of the enzyme.

Heat Stability of the Protease:

Since the 60,000 molecular weight protease appeared to predominate (Table II, #9), it was possible that this was the more stable of the two forms of the enzyme. Therefore, it was useful to study the heat stability of the two forms of the enzyme (i.e. Samples #9 A and #9 B from Table II, for the 60,000 and 30,000 molecular weight forms, respectively). An indication of the presence of multiple proteases might be seen if there were varying degrees of inactivation of proteolytic activity assayed at different pH values. The heat stabilities of samples #9 A and #9 B (Table II) were examined under various conditions (Fig. 14 and 15). Pretreatment of the protease samples with 10^{-2} M EDTA did not make them more susceptible to heat denaturation

Figure 12. Effect of temperature on protease activity. Assay mixtures contained 0.3 mg of enzyme per ml and 2.0 mg of casein (pH 6.7) per ml.

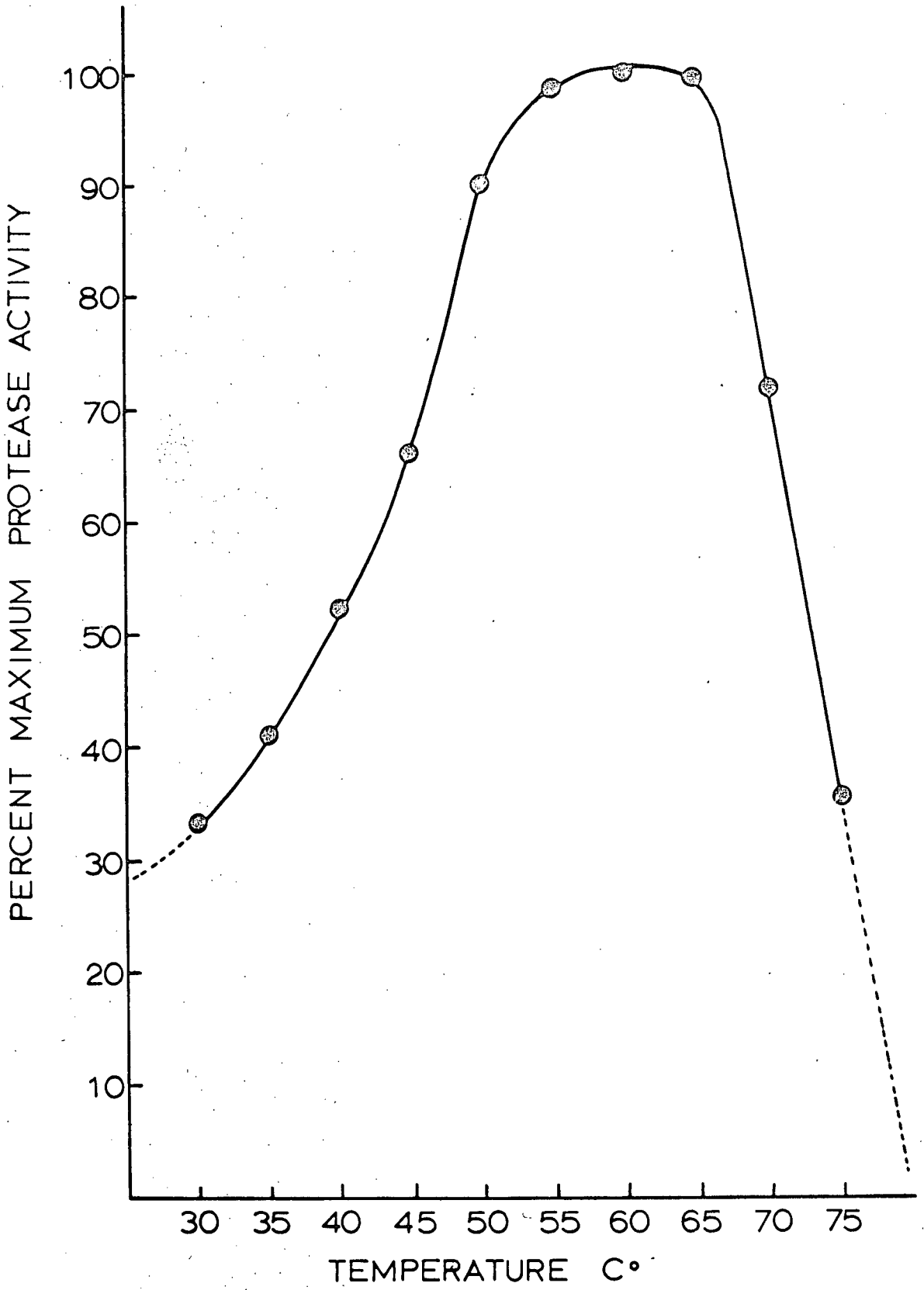

PERCENT MAXIMUM PROTEASE ACTIVITY
100
90
80
70
60
50
40
30
20
10
30 35 40 45 50 55 60 65 70 75
TEMPERATURE C°

Figure 13. Arrhenius plot of protease activity at pH 6.7.

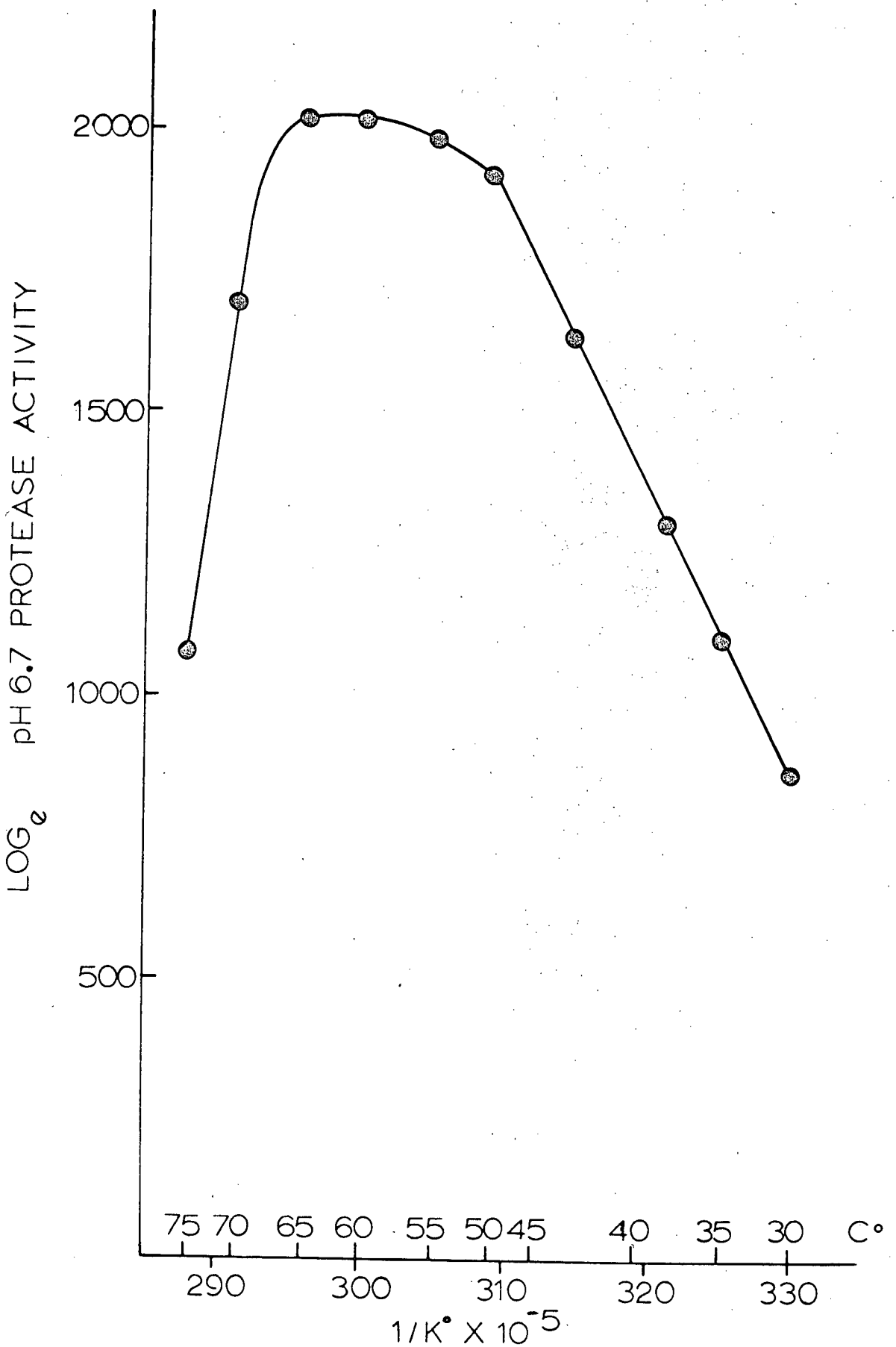

2000
1500
1000
500
LOG$_e$ pH 6.7 PROTEASE ACTIVITY
75 70 65 60 55 50 45 40 35 30 C°
290 300 310 320 330
1/K° X 10^{-5}

Figure 14. Heat stability of (Table II, #9 B). Enzyme protein was exposed to temperatures indicated for 15 min. Symbols: Calcium 10^{-3} M added to EDTA 10^{-2} M treated enzyme, (● - ●), EDTA treated enzyme, (■ - ■), and untreated enzyme, (▲ - ▲).

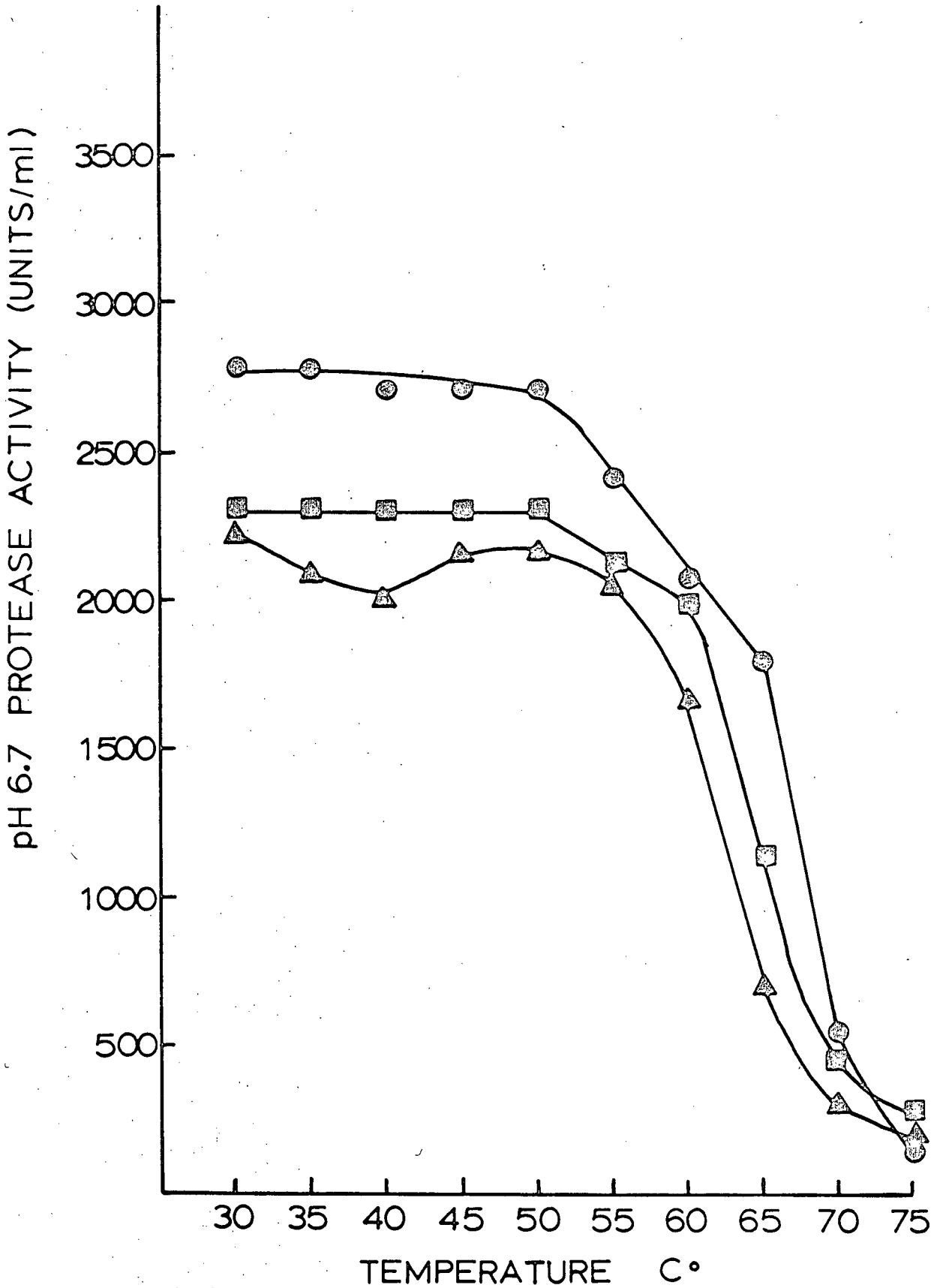
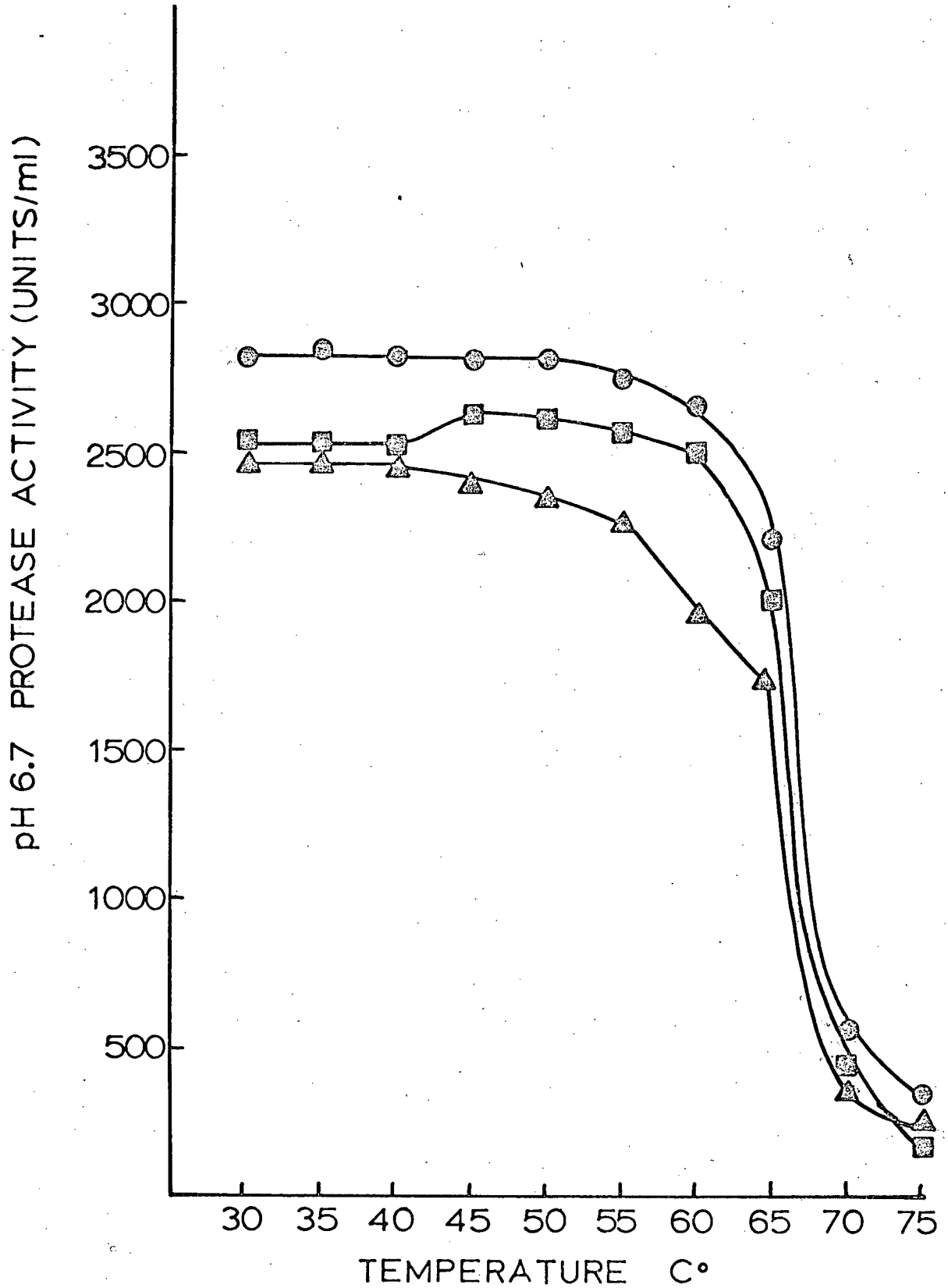

pH 6.7 PROTEASE ACTIVITY (UNITS/ml)
3500
3000
2500
2000
1500
1000
500
30
35
40
45
50
55
60
65
70
75
TEMPERATURE C°

Figure 15. Heat stability of (Table II, #9A).
Enzyme protein was exposed to temperatures indicated for 15 min.
Symbols: Calcium 10^{-3} M added to EDTA 10^{-2} M treated enzyme, (● - ●), EDTA treated enzyme, (■ - ■), and untreated enzyme, (▲ - ▲).


pH 6.7 PROTEASE ACTIVITY (UNITS/ml)
3500
3000
2500
2000
1500
1000
500
30
35
40
45
50
55
60
65
70
75
TEMPERATURE C°

but rather it gave an increase in proteolytic activity of 8.0 to 10%. This phenomenon could possibly be caused by the chelation of inhibitory metal ions by EDTA. The observation that chelating agents did not inhibit protease activity suggested that the protease was not a metalloenzyme. In this respect, the enzyme of B. amylophilus H-18 resembles the proteases of Aspergillus oryzae (Buguish, 1963), Paecilomyces variot: (Sawada, 1964) and Rhizopus chinensis: (Fukumoto et al 1967). Blackburn (1965) treated extracts of protease derived from disintegrated cells of B. amylophilus H-18 with 10^{-2} M EDTA and observed an 8.0% increase in activity of the protease. Addition of calcium ions (10^{-3} M) increased by 10% the activity of both the EDTA treated and untreated protease at lower temperatures. However, the temperature of irreversible denaturation of sample #8 (Table II) was equal for all four different pH values of protease activity, which were not the results to be expected if there were a number of proteases present, (Table IV).

Active Site Inhibition of the Protease:

The esterase and protease activities were compared in an effort to determine whether one or more enzymes were producing the proteolytic activity found between pH 4.5 and 11.5. Blackburn (1968 b) noted a change in the ratio of esterase activity: protease activity between various extracts obtained from disintigrated cells

Table IV. Heat denaturation of enzyme sample #8 (Table II) for varying lengths of time at 60 C.

Incubation Time at 60 C.	Percentage of Activity Remaining			
	pH 5.0	pH 6.0	pH 8.0	pH 11.0
Zero Time	100	100	100	100
10 minutes	100	91	82	84
20 minutes	82.0%	82.0%	79%	78.5%

of B. amylophilus H-18. Esterase activity in Sample #8 (Table II) against Nα Benzoyl-L-arginine methyl ester (BAME) was found to have a single optimum at pH 8.0 and TLCM at 10^{-3} M concentration completely inhibited this activity (Table V). Blackburn (1968 b) showed that a considerable proportion of the total protease activity may be due to an enzyme with a trypsin-like specificity and protease and esterase activities were inhibited by di-isopropyl-phosphofluoridate, a serine-protease inhibitor. A number of bacterial proteases with a trypsin-like activity have been described such as a streptococcal protease (Mycek et al,1952) and a semi-purified protease from Bacillus licheniformis (Hall et al, 1966). However, neither the esterase nor the protease were inhibited by soy-bean trypsin inhibitor, and if the main proteolytic activity is not due to a trypsin-like enzyme then neither is it like chymotrypsin nor pepsin as it did not attach substrates specific for these enzymes (Blackburn, 1968 b).

Protease activity was not completely inhibited by 10^{-3} M TLCM. Unlike the esterase activity which was inhibited 100% at pH 8.0, the protease was inhibited at pH 5.0, 6.7, 8.0 and 11.0, 20%, 29%, 30% and 48% respectively. It was evident from the difference in the extent of inhibition between the pH 5.0 and 11.0 activities that there may be different enzymes involved. The inhibition of the protease also indicates that the active site in the protease molecule contained a serine residue since TLCM binds specifically to serine.

Table V. Hydrolysis of Nα Benzoyl-L-arginine methyl ester (BAME) by an enzyme preparation (sample #8, Table II).

pH	μmole/mg protein/min (BAME)	μmole/mg protein/min (BAME) TLCM 10^{-3} M added
6.0	-	-
6.5	10.0	-
7.0	16.6	-
7.5	19.2	-
8.0	23.0	-
8.5	17.0	-
9.1	13.4	-
9.6	5.1	-
10.0	-	-

GENERAL DISCUSSION

The protease which was liberated in cultures containing late stationary phase cells of B. amylophilus H-18 was shown to be of relatively low molecular weight. Its purification was undertaken in anticipation that it could be more readily purified than the particle-bound protease which Blackburn (1968 b) liberated from disintegrated log phase cells of this organism. The relatively weak proteolytic activity found in the culture supernatant made it necessary to process large volumes. Fortunately, the proteolytic activity could be removed from the supernatant by absorbing it to DEAE-Sephadex. The activity could then be eluted from the washed absorbent in a concentrated and purified form.

Blackburn (1968 b) demonstrated two pH optima for B. amylophilus H-18 protease and suggested that two or more proteases might be present. In this present work, it was established that the culture supernatant possessed proteolytic activity over a wide pH range (pH 4.0 to 11.0) with peaks of activity at pH 5.0, 6.7, 8.0 and 11.0. Again, this suggested the presence of several different proteases, and the initial objective was to purify the pH 6.7 protease. It was found at each step in purification, by DEAE-Sephadex chromatography, gel filtration and isoelectric focusing that the protease activities at the different pH values, were not separated from each other nor did the ratio of their

activities change significantly. These results favoured the idea that all the proteolytic activity, over the pH range examined was due to a single protease. There were only two minor pieces of evidence against this hypothesis. In preliminary experiments, some fractions obtained from gel filtration of an ammonium sulfate precipitated protease showed differing ratios of proteolytic activities at pH 5.0, 6.7, 8.0 and 11.0. In addition, the activities at these pH values were inhibited to different extents, by treatment of the purified enzyme with TLCM.

In spite of this, gel electrophoresis of purified protease fractions failed to resolve those activities. In other words, a band showing the pH 6.7 proteolytic activity also gave peaks of activity at pH 5.0 or pH 11.0. The proteolytic activities at pH 5.0, 6.7, 8.0 and 11.0 were inactivated to the same extent by heat treatment.

The interesting observation was made that gel filtration could resolve the proteolytic activity into two fractions having molecular weights of approximately 30,000 or 60,000. Since the pH range of both proteolytic fractions was the same it seemed possible that the 60,000 molecular weight species was a dimer of the 30,000 molecular weight species. This was substantiated by a demonstration that the 30,000 molecular weight fraction on rerunning through the gel, gave a peak characteristic of the 60,000 molecular weight protease. Further evidence that the two molecular weight species existed in equilibrium was given by the fact that gel electrophoresis always demonstrated two characteristic protease bands. The only exception to this was where

poor resolution was attained, through the use of insufficiently long columns of acrylamide gel. It is proposed that the dimer was more stable than the monomer as the 60,000 molecular weight species predominated on gel filtration and the upper band on acrylamide gel electrophoresis was always greater in protein and protease activity.

Neither EDTA - nor 4.0 M urea treatment of the purified protease resulted in a predominance of the 30,000 molecular weight species. The conditions for splitting the dimer, if indeed that is what the 60,000 molecular weight material represented, remained unknown. More drastic methods of effecting a separation could not be used to the resulting inactivation of the protease.

Amino acid analysis of the protease revealed a complete absence of cysteine. It has been shown for several other extracellular proteases purified from bacteria that disulphide bridges are not involved in maintaining tertiary structure.

The temperature optimum for protease activity was between 60 C and 65 C, in spite of the temperature of inactivation of the enzyme being only 50 C. This could have been due to the casein used as substrate protecting the enzyme from denaturation during assay.

Desmazeand and Hermier (1968), working with a protease from Micrococcus caseolyticus, and Ryden and Hofsten (1968) who were examining a protease from Serratia indica, found that EDTA at 10^{-3}M concentration inhibited proteolytic activity. When the protease from B. amylophilus H-18 was treated with 10^{-3}M EDTA a 8 - 10%

increase in proteolytic activity was observed. The heat stability of the enzyme was not affected by addition of EDTA. This suggested that the protease was not a metalloenzyme. Although the addition of Ca^{++} increased, the activity of the protease was not dependant upon this ion for its activity. Although the proteases produced by various species of Micrococcus were reported not to require Ca^{++} for activity, they were activated by this ion (Colbert, 1957 and Gomer, 1950).

The purified protease still showed esterase activity, which was maximal at pH 8.0. It was significant that although esterase activity was inhibited completely by 10^{-3}M TLCM, protease activity was not completely inhibited under these conditions. This observation again suggested the possibility of more than one protease being present, even in highly purified preparations.

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