PRODUCTION AND PROPERTIES OF A PROTEASE SECRETED BY *Pseudomonas fluorescens R8*

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To my parents; my wife, Rokya; my son and daughter, Mohamed and Fatma, I dedicate this thesis.

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SUMMARY

Eight genera of protease-producing psychrotrophic bacteria were isolated from a number of East of Scotland farms. Four genera were found to be Gram-positive and four were Gram-negative. The Gram-positive genera were Bacillus spp, Micrococcus spp, Corynebacterium spp and Staphylococcus spp. The Gram-negative genera were Flavobacterium spp, Pseudomonas spp, Acinetobacter spp and Cytophaga spp. Pseudomonas fluorescens represented 42.9% of the Pseudomonas spp isolated. Pseudomonas fluorescens R8 was the most active proteolytic strain when grown on standard methods agar supplemented with 10% reconstituted skim milk.

P. fluorescens R8 secreted a constitutive protease, the production of which was not highly affected by the presence of inducers. This strain utilised glutamate as a sole source of carbon and nitrogen and secreted protease either in the presence or absence of $CaCl_2$. Protease production was detected at the beginning of growth in all the tested media. However, maximum production of the enzyme (EU growth^{-1}) was found to be either at the end of exponential phase or the beginning of stationary phase. Enzyme protein (µg ml^{-1}) increased about 2.4 fold throughout the growth cycle when strain R8 was grown in glucose basal medium in the absence of $CaCl_2$. In the presence of $CaCl_2$ however the increase was about 6.2 fold. Enzyme protein per growth (EP growth^{-1}) in glucose basal medium containing $CaCl_2$ was about 10 times higher than when $CaCl_2$ was omitted, when measured at the beginning of stationary

phase.

An extracellular protease from Pseudomonas fluorescens R8 was purified to electrophoretic homogeneity in three steps. This protease was a major component in the culture supernatant. Two peaks were eluted after ion exchange chromatography. Peak A contained about 60% of the total activity whereas peak B contained about 15%.

The two peaks were found to have the same molecular weight (45K daltons) and their immunochemical identity has been determined. Characterisation of peak A showed it to be a metallo-alkaline protease which contained calcium and zinc. The enzyme had an optimum temperature around 40°C and optimum pH ≈ 7.5. The activity of the protease was inhibited by o-phenanthroline, EDTA and EGTA. It was more sensitive to o-phenanthroline than EDTA and EGTA. The amino acid composition showed that the enzyme contained high levels of aspartic acid and glycine but lacked cysteine. The total carbohydrate content was very low revealing that the protease was not a glycoprotein.

The protease was heat-stable, the D-value at 140°C was 1 min and 28% of the starting activity remained after heating at 74°C for 17 sec. Heating the enzyme at 55°C led to autolysis. The enzyme attacked casein, its fractions, bovine serum albumin and cytochrome c. It also attacked β-lactoglobulin.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

CHAPTER 1

INTRODUCTION

1.1 Characterisation and properties of psychrotrophic bacteria

1.1.1 Psychrotrophic microorganisms in the dairy industry

The widespread practice of bulk collection of farm refrigerated milk and storage of milk at 3-8°C at the dairy plant has resulted in new quality control problems related to the growth and metabolic activity of pyschrotrophic bacteria (Richardson & Te Whaiti, 1978; Law, 1979; Cousin, 1982; Yan et al., 1985; Andrews, 1986).

Milk is collected from the farms every other day in most areas of the USA (Cousin, 1982) and five times in two weeks in the Netherlands (Mol & Vincentie, 1981). For economic and social reasons milk may be stored at the processing plant for three to four days before it is processed (Muir et al., 1978; Cousin, 1982). A similar situation exists in the UK and most milk producing countries. Milk is kept at refrigeration temperatures before processing (Thomas, 1974a; Cousins et al., 1977). The extended storage of raw milk prior to processing in the plant is selective for the growth of psychrotrophic bacteria (Thomas, 1970; Thomas & Thomas, 1973a,b; Cousin, 1982; Patel et al., 1986).

1.1.2 Definition of psychrotrophic bacteria

As early as 1887, Forster observed bacterial growth at 0°C (Cousin, 1982). Microorganisms which were capable of growing at low temperatures were first termed "psychrophiles" by Schmidt-Nielsen (1902). He defined "psychrophiles" as cold-loving bacteria which were able to grow at 0°C (Ingraham & Stokes, 1959; Ingraham, 1962; Thomas,

1966; Thomas & Thomas, 1973a; Cousin, 1982). This definition is derived from the Greek words Psychros, which means cold, and philes, which means loving. Two other terms "cryophile" and "rhigophile" derived from Greek words, have been used to describe microorganisms that grow at low temperatures (Ingraham & Stokes, 1959; Cousin, 1982). These terms have been rejected by several researchers, because they imply a preference for growing at low temperatures when the optimum temperature for growth of these microorganisms is often around 20°C (Ingraham, 1962; Thomas & Druce, 1969; Thomas & Thomas, 1973a).

As described above the term psychrophile defines those bacteria with a low optimum growth temperature. This is an inaccurate description of microorganisms which are capable of growth at low temperatures.

Terms such as Psychrocantericus or cold-conquering, Psychrotolerant or cold-tolerant and Eurythermic or capable of growing over a wide temperature range have been put forward, but none of these terms have gained acceptance (Eddy, 1960; Stokes, 1963; Kandler, 1966; Thomas et al., 1966). Eddy in 1960 introduced the term "psychrotrophic" or "cold-thriving" for those bacteria which are able to grow at 5°C or below regardless of their optimum growth temperature. He suggested that the term "psychrophiles" be used only when a low optimum growth temperature is implied.

At the International Dairy Federation seminar on "Psychrotrophic Organisms in Milk and Dairy Products" in 1968 the following definitions were adopted:

i) In the dairy industry, psychrotrophs are defined

as those microorganisms which can multiply at a temperature of 7°C or less, irrespective of their optimum growth temperature.

ii) Psychrophiles are those psychrotrophic organisms having an optimum growth temperature below 20°C (International Dairy Federation Annual Bulletin, 1969).

1.1.3 Types of psychrotrophic microorganisms

Psychrotrophs found in milk are mainly bacteria. Some psychrotrophic moulds (Aspergillus; Cladosporium; Geotrichum and Penicillium) and yeasts (Candida; Saccharomyces; Rhodotorula; Torulopsis and Trichosporon) have been isolated and found to be associated with defects and off-flavours in butter and cream (Thomas, 1970; Thomas & Druce, 1971; Cousin, 1982).

Psychrotrophic bacteria make up less than 10% of the total viable count of good quality fresh bulk tank milk (Thomas & Thomas, 1973a; Cousins & Bramley, 1981), but can represent one-third of the total bacterial content of such milk after only two days of storage at refrigeration temperatures (Mourgues & Auclair, 1965). La Grange & Nelson (1961) found that in poor quality bulk tank milk psychrophiles formed 75% or more of the total bacterial content.

Most of the raw milk psychrotrophs are Gram-negative rods. However, some Gram-positive psychrotrophs have also been isolated, but are usually present in smaller numbers than the heat-sensitive Gram-negative rods (Witter, 1961; Shehata & Collins, 1971; Cousin, 1982; Malik & Mathur, 1983). Members of the genus Pseudomonas are usually

dominant, making up to 45% of the proportion of psychrotrophic flora. The second largest proportion is constituted by Achromobacter spp (Juffs, 1973a; Malik & Mathur, 1983).

Among the genus Pseudomonas the most common species isolated from refrigerated raw milk is P. fluorescens although its apparent high incidence may be due to the ease with which it can be identified rather than to its true distribution (Law, 1979). Juffs (1973a) found that P. fluorescens accounted for 75% of all Pseudomonas spp isolated from Australian raw and pasteurised milk. Pseudomonas spp are important not only because they dominate the psychrotrophic bacteria, but also because of their ability to produce extracellular proteases and lipases (Law, 1979). Although raw milk psychrotrophs are generally killed by proper pasteurisation, their extracellular proteases and lipases, being heat-stable, can survive and adversely affect the quality of dairy products (Cogan, 1977; Law, 1979; Mikolajcik , 1979; Stepaniak et al., 1987). Banerjee & Black (1986) isolated 34 psychrotrophic bacteria from milk and milk products; they found that 22 strains were Pseudomonas spp, 10 were Enterobacteriacea spp, one Aeromonas hydrophila and one Bacillus cereus.

Of the Gram-positive bacteria, Bacillus species in particular have been isolated from pasteurised milk stored at low temperatures. Psychrotrophic strains of B. cereus, B. licheniformis and B. lentus were also isolated from pasteurised dairy products (Grosskopf & Harber, 1969; Shehata & Collins, 1971; Credit et al., 1972; Johnston &

Bruce, 1982). Mikolajcik (1979) found other species including B. subtilis, B. circulans, B. coagulans, B. macerans, B. polymxa, B. firmus and B. licheniformis. Of all species isolated from raw milk and dairy products, Bacillus cereus was of major concern since it caused a "bitty cream" defect and food poisoning (Overcast & Atmaram, 1974; Cox, 1975; Mikolajcik, 1978; Coghill & Juffs, 1979).

Table 1.1.1 lists proteolytic psychrotrophic microorganisms isolated from raw milk and dairy products (modified after Suhren, 1983).

1.1.4 Sources of psychrotrophic bacteria

Psychrotrophs have been isolated from soil, vegetables, meat, fish, milk, flour and air (Ingraham & Stokes, 1959). Soil, water, plants and animals form the natural habitats of these organisms (Stokes & Redmond, 1966; Thomas & Thomas, 1973a). Psychrotrophic bacteria are rarely present in the udder, they enter the milk from soil, water and poorly cleaned dairy equipment (Thomas et al., 1966; Thomas & Thomas, 1978; Cousin, 1982).

1.1.5 Characterisation of pyschrotrophic bacteria

The characteristics that have been used to identify psychrotrophic isolates are Gram reaction, cell shape, motility and standard biochemical tests (Orr et al., 1964; Cowan & Steel, 1974; Hendrie & Shewan, 1979; Malik & Mathur, 1983). In 1979, Otte et al. using a microtiter primary test identified bacteria to the genus level using Gram stain, morphology, motility, acid fastness, spore formation, catalase and oxidase tests and oxidation/fermentation test. These tests were used to identify most of

Table 1.1.1 Proteolytic psychrotrophic microorganisms isolated from raw milk (after Suhren, 1983)

Isolate	Reference
Pseudomonas spp	Bengtsson et al. (1973) Adams et al. (1975) Cousin & Marth (1977b,d) De Beukelar et al. (1977) Miura et al. (1977) Richardson & Te Whaiti (1978) Richter (1979) Gebre-Egziabher et al. (1980a) Malik & Mathur (1983)
P. fluorescens	Skean & Overcost (1960) Juffs (1973 and 1974) Drissen & Stadhouders (1974) Law et al. (1979b) Milliere & Veillet Poncet (1979)
P. putrefaciens	Van der Zant (1957) Skean & Overcast (1960)
P. fragi	Skean & Overcast (1960) Law et al. (1979b)
P. aureofaciens	Law et al. (1979b)
P. putida	Law et al. (1979b)
P. aeruginosa	Purschel & Pollack (1972)
Acintobacter spp	De Beukelar et al (1977) Law et al. (1979b)
Acromobacter spp	Purschel & Pollock (1972) Richter (1979)
Aeromonas spp	Purschel & Pollack (1972) Richardson & Te Whaiti (1978) Law et al. (1979b)
Alcaligenes spp	Malik & Mathur (1983)
Enterobacter liquefaciens	Hartmann & Hastings (1972)
Escherichia freundii	Nakajima et al. (1974)
Flavobacterium spp	Cousin & Marth (1977b,d) Miura et al. (1977) Richardson & Te Whaiti (1978) Malik & Mathur (1983)
Xanthomonas spp	Law et al. (1979b)

Table 1.1.1 (cont'd)

Isolate	Reference
Cytophaga spp	Law et al. (1979b) Milliere & Veillet Poncet (1979)
Proteus spp	Law et al. (1979b)
Micrococcus spp	Malik & Mathur (1983)
Staphylococcus spp	Malik & Mathur (1983)

the strains isolated from milk and dairy products.

Analysis of the base composition of DNA of a large number of strains of Pseudomonas spp indicated that the guanine + cytosine content ranged from 58 to 69 mol % (Palleroni, 1975). However there is always overlap and using another approach such as nucleic acid hybridisation would be more accurate in characterising psychrotrophic bacteria. Lee in 1977 indicated that certain diagnostic tests are useful in the separation of pseudomonads into five phenons and thus showed that estimation of the guanine + cytosine content of the DNA is not always necessary.

In many circumstances, particularly in the medical field, a rapid method for identification of unknown isolates is necessary. For the common medical bacteria, Cowan & Steel (1974) devised a set of diagnostic tables to identify the isolated unknown. King & Philips (1978) have applied simple biochemical techniques appropriate mainly for clinical pseudomonads and related bacteria. Organisms were subdivided into groups on the basis of three tests, namely the glucose oxidation-fermentation test and tests for oxidase activity and motility. Identification of bacteria is time-consuming and therefore there is great demand for quick identification procedures. Multitest systems have been developed for rapid identification. These microtechniques which incorporate a number of media in a single unit enable the researcher to perform a large number of biochemical tests on a single bacterial colony in a short time.

In 1925 Buchanan took the physiological concepts along

with the morphological ones, to form the basis of a classification for the genus Pseudomonas in the first edition of Bergey's Manual of Determinative Bacteriology. Rhodes (1959) in his investigation using 169 isolates, mainly fluorescent strains, arrived at an extensive description of the genus Pseudomonas and he revised the definition of Pseudomonas fluorescens. Shewan et al. (1960) carried out a series of tests on the basis of which broad groupings of microorganisms were obtained. This scheme of identification was devised primarily for identification of strains isolated from fresh and spoiled fish. The tests used in this work are similar to those mentioned in the tables used by Cowan & Steel (1974).

Kling (1960) using a variety of morphological and biochemical tests divided the isolated unknowns into three species - P. fluorescens, Pseudomonas aeruginosa and Pseudomonas putida. In addition he defined a more extensive set of characteristics to identify the genus Pseudomonas.

Stanier et al. (1966) subjected a varied collection of 267 isolates to detailed study, using biochemical, physiological and nutritional characteristics. The definition obtained from this study of the aerobic pseudomonads revealed similar characteristics to those mentioned by other investigators. The pseudomonads were shown to have a very wide-ranging nutritional spectrum and could utilise a wide range of simple organic compounds as sole carbon and energy sources.

In the study carried out by Stanier et al. (1966) primary differentiation of the 267 Pseudomonas strains

examined was made into three subgeneric groups - fluorescent, acidovorans and alcaligenes. The most complex of these groups is the fluorescent one which contains three species, P. aeruginosa, P. fluorescens and P. putida. P. aeruginosa showed a high degree of internal phenotypic uniformity, whereas P. fluorescens and P. putida could be subdivided into several distinct biotypes. Division into the three subgenetic groups and into species was made on the basis of an extensive number of tests which the authors considered only as a minimal level. This work has played a primary role in reshaping and defining the pseudomonads at the genus and species level. The work by Stanier et al (1966) is taxonomically important, but has not produced a quick, concise identification scheme.

Samagh & Cunningham in 1972 isolated 653 alcaligenic and psychrotrophic bacteria from 136 samples of milk and dairy products. The 182 isolates belonging to the genus Pseudomonas were further subdivided into four groups. They found that 178 isolates of the Pseudomonas strains belonged to the fluorescent group as defined by Stanier et al. (1966). Molin & Ternström (1982) isolated 200 psychrotrophic pseudomonads. These strains were numerically studied by 174 biochemical and physiological tests. They clustered into 15 groups, of which nine were regarded as major clusters.

The 8th Edition of Bergey's Manual of Determinative Bacteriology has reduced the recognised number of Pseudomonas to 29 species. However, an additional 236 species are listed which have been incompletely described. The

latest edition of the Bergey's Manual divides the genus Pseudomonas into five sections: the first four are almost the same as in the eighth edition, but the fifth section has 62 species whose relationships to the above groups were unknown until now (Bergey's Manual of Systematic Bacteriology, Vol. I, 1984).

1.2 Effect of psychrotrophic bacteria and their enzymes on milk and dairy products

1.2.1 Introduction

Many pyschrotrophic bacteria which grow in milk and dairy products secrete extracellular proteases, lipases and phospholipases. The presence of these enzymes can cause problems for the dairy industry: the appearance of off-flavours, a decrease in keeping quality or changes in the behaviour of milk during manufacture of cheese and other dairy products.

1.2.2 Effect of proteases from psychrotrophic bacteria on milk

Proteases from psychrotrophic bacteria cause changes in milk proteins, which affect the keeping quality of milk and its products. Many investigators have shown that most raw milk supplies contain heat-stable proteases or bacteria able to secrete them (Adams et al., 1975; Speck & Adams, 1976; Cogan, 1977; Mitchell et al., 1986; Patel et al., 1986). Mottar (1981) reported that it is the proteolytic count, not the total number of bacteria in raw milk which can be correlated with proteolytic activity in UHT milk.

Organoleptic defects were detected when the number of psychrotrophs were in the range 5 x 10^6-10 x 10^6 CFU/ml and after the maximum stationary growth phase was completed (Thomas & Thomas, 1973a,b). The populations of various psychrotrophs (per ml) found to cause off-flavours were as follows: Pseudomonas spp. 5.2-100 x 10^6; Alcaligenes spp. 2.5-14 x 10^6; Flavobacterium spp. 8.3-120 x 10^6 and yeasts 2.5-14 x 10^6 (Tekinson & Rothwell, 1974).

UHT milk theoretically should have a shelf life of up to 12 months. However, it has been found that after only three months spoilage such as gelation triggered by the action of heat-stable proteases can occur (Adams et al., 1976; Richardson & Newstead, 1979). The presence of thermoresistant proteases could restrict the widespread use of UHT milk (Adams et al., 1975; Speck & Adams, 1976).

Law et al. (1977) working with P. fluorescens AR_{11} reported that gelation in UHT-sterilised milk was due to the extracellular protease rather than to cell-bound or intracellular proteases. UHT-sterilised milk was more sensitive towards proteases from P. fluorescens than raw milk (McKellar, 1981); this could be due to the destruction of protease inhibitors or the effect of high temperature which may alter the susceptibility of the caseins towards the bacterial proteases.

Proteases from psychrotrophs have been reported to attack k-casein preferentially, which is responsible for stabilising the colloidal casein micelles (Law et al., 1977; Grieve & Kitchen, 1985). This leads to the formation of para-k-casein, which results in the formation of a

coagulum in a way similar to that caused by the action of chymosin. Milk which had extensive k-casein proteolysis coagulated during UHT processing (Adams et al., 1976). β-Casein was found to be hydrolysed more rapidly than α-casein (Snoeren et al., 1979; Stepaniak et al., 1982a; Tayfour et al., 1982).

The degree of proteolysis in milk caused by proteases from psychrotrophs has been studied by measuring nitrogen components released from caseins. Nakanishi & Tanabe (1970) found that casein nitrogen decreased, whereas non-protein nitrogen increased during the storage of milk. An increase in both non-casein nitrogen and non-protein nitrogen was also reported when cold stored milk was treated with Pseudomonas spp protease (Adams et al., 1975). Increasing protease concentration leads to bitter flavour (Richardson & Newstead, 1979).

Burlinghame-Frey & Marth (1984) inoculated raw milk with a proteolytic psychrotrophic culture of Lactobacillus spp. They found that the casein micelles were reduced in size due to the proteases secreted by the psychrotrophs. The reduction in size was related to the incubation time.

By contrast, whey proteins have been shown to be relatively insensitive to the action of proteases from psychrotrophs. This is possibly due to the highly globular nature of whey proteins unlike the random non-helical structure of caseins (Law et al., 1977; Bengtsson et al., 1973; Stepaniak et al., 1982a; Fairbairn & Law, 1986a).

About 25% of the deteriorative changes in milk are caused by growth and metabolic activity of thermoduric

psychrotrophic bacteria (Tekinson & Rothwell, 1974; Tinuoye & Harmon, 1975). Organoleptic defects were noted when the population of these bacteria reached 3-4 x 10^6 CFU/ml in milk held at 7.2°C for 6 d. Sweet curdling defects in pasteurised milk was caused by B. cereus. This curd usually appears at the bottom of the bottle and may result from casein breakdown (Overcast & Atmaram, 1974; Whasam et al., 1977; Mikolajcik, 1978).

1.2.3 Effect of proteases from psychrotrophic bacteria on cheese

Cheese is the dairy product most affected by the presence of psychrotrophs or their proteases (Table 1.2.1) (Cogan, 1977). This is probably due to the concentration of the casein during the manufacture of cheese and the ripening period before consumption.

The effect of psychrotrophs and their proteases on the rennet coagulation time has given contradictory results. When cheese milk was stored for long periods at low temperatures, the coagulation time by chymosin increased (Ritter, 1970; Vitagliano et al., 1971; Cerna et al., 1974). It was increased by 11% when milk was stored for three days at 2-3°C (Antila, 1971). Ritter (1970) found that adding calcium salts or acidification can overcome the increase of the rennetting time. In contrast, Cousin & Marth (1977e) found that rennet ing was reduced when psychrotrophic bacteria were previously grown in the milk. The decrease in coagulation time was probably due to the degradation of β-and α-caseinS by proteases from psychrotrophic bacteria which may make k-casein more accessible to coagulation by

Table 1.2.1 Effect of crude protease from Pseudomonas fluorescens P26 on the keeping quality of milk and some dairy products (Cogan, 1977)

Product	Time and temperature of storage	Units of protease /ml of cream, mix or milk	Conditions of incubation of protease	Effect of the enzyme
Milk	Unspecified time at 7°C	7.9	12 h at 4°C	No specific effect on flavour
Milk	Unspecified time at 7°C	7.9	12 h at 4°C before pasteurisation at 68°C for 30 min	No effect on flavour
Cheddar cheese	28 days at 7°C	0.94	12 h at 4°C	Significantly lower flavour score in cheese made from proteinase-treated milk but tyrosine level negatively correlated with flavour
Cottage cheese	28 days at 10°C	0.86	12 h at 4°C	As for cheddar cheese
Butter	30 days at 4°C	11.0	12 h at 21°C before heating to 72°C for 30 min	No effect on flavour but tyrosine negatively correlated with flavour
Ice cream mix	15 days at 7°C	8.4	Overnight at 4°C before heating to 68°C for 30 min	No effect on flavour but tyrosine level increased about two-fold in proteinase treated mix

rennet.

One further effect of proteases on cheese milk was observed by Cousin & Marth (1977c,d). They found that previous growth of psychrotrophs in milk resulted in an increase of lactic acid produced by Streptococcus lactis, Streptococcus cremoris, Streptococcus thermophilus and Lactobacillus bulgaricus. This increase was due to the proteolysis of milk proteins which supply lactic acid bacteria with low molecular weight nitrogen compounds released by the degradation of casein.

Some investigators found that the quality of cheese made from cold stored milk was inferior to the control cheese in terms of texture and flavour and concluded that these differences were due to the action of heat-stable proteases (Ohren & Tuckey, 1969; Bottazzi, 1970; Scott, 1972; Cousin & Marth, 1977b). Other studies showed that Cheddar cheese made from milks in which Pseudomonas and Flavobacterium species had been growing took less time to manufacture. The curds were firmer and the taste was unacceptable after six months' ripening at 10°C when compared with uninoculated control cheeses (Cousins & Marth, 1977b). However, others have reported that there were no problems using cold stored milk in making cheese (Annibaldi et al., 1975).

Psychrotrophs and their proteases may also have some effect on the yield of cheese. Feuillat et al (1976) reported a 5% decrease in soft cheese yield when cheese was made with heat-treated high psychrotrophic count milks. The decrease in cheese yield was due to the increase of

non-protein nitrogen because of hydrolysis of casein into small peptides (Allauddin et al., 1976). This decrease in yield is of economic significance and considerable financial losses can occur (Stofer & Hicks, 1983).

A slight breakdown of β- and k-casein was observed when raw milk contains 1 x 10^7 CFU/ml of Pseudomonas and Acinetobacter species. However, this slight breakdown was not enough to decrease the yield of cheese manufactured from this milk (Law et al., 1979a). Using cell-free filtrates from a proteolytic species of Bacillus to accelerate ripening of Ras cheese, Magdoub et al. (1979) found that the ripening period was reduced by 50% and the cheese quality was equal to or better than the control. Cheddar cheese which had been manufactured using proteases from Pseudomonas species to coagulate the milk had an unclean and bitter flavour and the body of the cheese was soft (Juffs, 1974).

Finally psychrotrophic bacteria may also be responsible for the surface spoilage of cottage cheese and the appearance of gelatinous or slimy curd. This is normally accompanied by bitter flavour (Thomas, 1958).

The above evidence leads to the conclusion that heat stable proteases, resistant to UHT processing play a significant role in the reduction of shelf life and keeping quality of milk and dairy products. Table 1.2.1 summarises the effect of Pseudomonas fluorescens P26 on the keeping quality of milk and some dairy products (Cogan, 1977).

1.2.4 Proteolytic enzymes and bitter flavour

The appearance of bitter flavour in milk and its

products is one of the organoleptic defects that can be caused by heat stable proteases secreted by psychrotrophic bacteria. Bitter peptides can also be released from casein by the action of proteolytic enzymes from rennet or starter bacteria (Stadhouders & Hup, 1975; Stadhouders et al., 1983). A protease from Kiwi fruit (Actimidia chimensis) has been shown to produce bitter peptides from milk proteins (Bachmann & Farah, 1982).

The most important factor in determining whether a peptide will be bitter or not is the degree of hydrophobicity (Clegg et al., 1974). According to the Bigelow scale (Bigelow, 1967) bitter peptides have an average hydrophobicity of > 1400 (cal/res); so these peptides contain a high proportion of amino acids with hydrophobic side chains (Giugoz & Solms, 1974; Visser et al., 1983).

The bitterness of peptides is assessed normally by a tasting panel. Bitter tasting standards are given to the tasters in order to determine the sensitivity to bitter compounds. Grading of sample peptide is carried out by comparison to standard bitter peptides (Schalinatus & Behnke, 1975a,b).

The primary structure of α_{S1}-casein and β-casein genetic variant A_2 is shown in Figures 1.2.1 and 1.2.2 (Eigel et al., 1984). Table 1.2.2 shows the amino acid sequence of different bitter peptides isolated from individual caseins and total caseins digested experimentally as well as cheeses which had developed flavour defects during manufacture and ripening (after Visser et al., 1975).

Most bitter peptides contained different arginine or

H_2N-ARG-PRO-LYS-HIS-PRO-ILE-LYS-HIS-GLN-GLY-LEU-PRO-GLN-
GLU-VAL-LEU-ASN-GLU-ASN-LEU-LEU-ARG-PHE-PHE-VAL-ALA-PRO-
PHE-PRO-GLN-VAL-PHE-GLY-LYS-GLU-LYS-VAL-ASN-GLU-LEU-SER-
LYS-ASP-ILE-GLY-SERP-GLU-SERP-THR-GLU-ASP-GLN-ALA-MET-
GLU-ASP-ILE-LYS-GLN-MET-GLU-ALA-GLU-SERP-ILE-SERP-SERP-
SER-GLU-GLU-ILE-VAL-PRO-ASN-SERP-VAL-GLU-GLN-LYS-GLU-ASP-
VAL-PRO-SERP-GLU-ARG-TYR-LEU-GLY-TYR-LEU-GLU-GLN-LEU-LEU-
LYS-LYS-TYR-LYS-VAL-PRO-GLN-LEU-GLU-ILE-VAL-PRO-ASN-SERP-
ALA-GLU-GLU-ARG-LEU-HIS-SER-MET-LYS-GLU-GLY-ILE-HIS-ALA-
GLN-GLN-LYS-GLU-PRO-MET-ILE-GLY-VAL-ASN-GLN-GLU-GLU-LEU-
ALA-TYR-PHE-TYR-PRO-GLU-LEU-PHE-ARG-GLN-PHE-TYR-GLN-LEU-
ASP-ALA-TYR-PRO-SER-GLY-ALA-TRP-TYR-TYR-VAL-PRO-LEU-GLY-
THR-GLN-TYR-THR-ASP-ALA-PRO-SER-PHE-SER-ASP-ILE-PRO-ASN-
PRO-ILE-GLY-SER-GLU-ASN-SER-GLU-LYS-THR-THR-MET-PRO-LEU-
TRP-COOH

Figure 1.2.1 Primary structure of bovine α_{S1}-casein (Eigel et al., 1984)

H_2N-ARG-GLU-LEU-GLU-GLU-LEU-ASN-VAL-PRO-GLY-GLU-ILE-VAL-
GLU-SERP-LEU-SERP-SERP-SERP-GLU-GLU-SER-ILE-THR-ARG-ILE-
ASN-LYS-LYS-ILE-GLU-LYS-PHE-GLN-SERP-GLU-GLU-GLN-GLN-GLN-
THR-GLU-ASP-GLU-LEU-GLN-ASP-LYS-ILE-HIS-PRO-PHE-ALA-GLN-
THR-GLN-SER-LEU-VAL-TYR-PRO-PHE-PRO-GLY-PRO-ILE-PRO-ASN-
SER-LEU-PRO-GLN-ASN-ILE-PRO-PRO-LEU-THR-GLN-THR-PRO-VAL-
VAL-VAL-PRO-PRO-PHE-LEU-GLN-PRO-GLU-VAL-MET-GLY-VAL-SER-
LYS-VAL-LYS-GLU-ALA-MET-ALA-PRO-LYS-HIS-LYS-GLU-MET-PRO-
PHE-PRO-LYS-TYR-PRO-VAL-GLN-PRO-PHE-THR-GLU-SER-GLN-SER-
LEU-THR-LEU-THR-ASP-VAL-GLU-ASN-LEU-HIS-LEU-PRO-PRO-LEU-
LEU-LEU-GLN-SER-TRP-MET-HIS-GLN-PRO-HIS-GLN-PRO-LEU-PRO-
PRO-THR-VAL-MET-PHE-PRO-PRO-GLN-SER-VAL-LEU-SER-LEU-SER-
GLN-SER-LYS-VAL-LEU-PRO-VAL-PRO-GLU-LYS-ALA-VAL-PRO-TYR-
PRO-GLN-ARG-ASP-MET-PRO-ILE-GLN-ALA-PHE-LEU-LEU-TYR-GLN-
GLN-PRO-VAL-LEU-GLY-PRO-VAL-ARG-GLY-PRO-PHE-PRO-ILE-ILE-
VAL-COOH

Figure 1.2.2 Primary structure of bovine β-casein genetic variant A_2 (Eigel et al., 1984)

Table 1.2.2 Bitter peptides isolated from caseins and cheeses (after Visser *et al.*, 1975)

Amino acid sequence	Casein fraction and residue number	Source	Reference
Gln-asp-lys-Ile-his-pro-phe-ala-gln-thr-gln-ser-leu-val-tyr-pro-phe-pro-gly-pro-ile-pro	β 46-67	Cheddar cheese	Hamilton *et al.* (1974)
Ala-gln-thr-gln-ser-leu-val-tyr-pro-phe-pro-gly-pro-ile-pro-asn-ser-leu-pro-gln-asn-ile-pro-pro-leu-thr-gln	β 53-79	Whole casein + papain	Clegg *et al.* (1974)
Pro-phe-pro-gly-pro-ile-pro-asn-ser	β 61-69	Cheese "Butter-kase"	Huber & Klostermyer (1974)
Ala-pro-lys	β 103-105	β-Casein + CNBr and trypsin subsequently	Pelissier *et al.*(1974)
Phe-leu-leu	β 190-192	β-Casein + cryst·rennin	Pel issier *et al.* (1974)
Tyr-gln-gln-pro-val-leu-gly-pro-val-arg-gly-pro-phe-pro-ile	β 193-207	Whole casein + rennet	Visser *et al.* (1975)
		Gouda cheese made with *S. cremoris* (HP)	Visser *et al.* (1983)
Tyr-gln-gln-pro-val-leu-gly-pro-val-arg-gly-pro-phe-pro-ile-ile	β 193-208	Whole casein + rennet	Visser *et al.* (1975)
		Gouda cheese + rennet	Visser *et al.* (1983)

Table 1.2.2 (cont'd)

Amino acid sequence	Casein fraction and residue number	Source	Reference
Tyr-gln-gln-pro-val-leu-gly-pro-val-arg-gly-pro-phe-pro-ile-ile-val	β 193-209	β-Casein + rennet	Visser et al. (1975)
		Gouda cheese made with S. cremoris (HP)	Visser et al. (1983)
Gln-gln-pro-val-leu-gly-pro-val-arg-gly-pro-phe-pro-ile-ile-val	β 194-209	Milk cultures from S. cremoris (strain HP)	Gordon & Speck (1965)
Arg-gly-pro-phe-pro-ile-ile-val	β 202-209	Whole casein + alk proteinase (B. subtilis)	Minamiura et al. (1972)
Gly-pro-phe-pro-ile-ile-val	β 203-209	β-Casein + trypsin	Matoba et al. (1970)
			Pelissier et al. (1974)
Phe-phe-val-ala-pro-phe-pro-gly-val-phe-gly-lys	α_{S1} 23-33	Trypsin digest of total casein	Matoba et al. (1970)
*Glu-leu-val-asn	α_{S1} 14-17	Bitter cheese	Hodges et al. (1972)
*Glu-asn-leu-leu	α_{S1} 18-21	Bitter cheese	Hodges et al. (1972)
Ala-pro-phe-pro-gln-val-phe	α_{S1} 26-32	Bitter cheese	Hodges et al. (1972)
Leu-trp	α_{S1} 198-199	Bitter Swiss mountain cheese	Guigoz & Solims (1974)

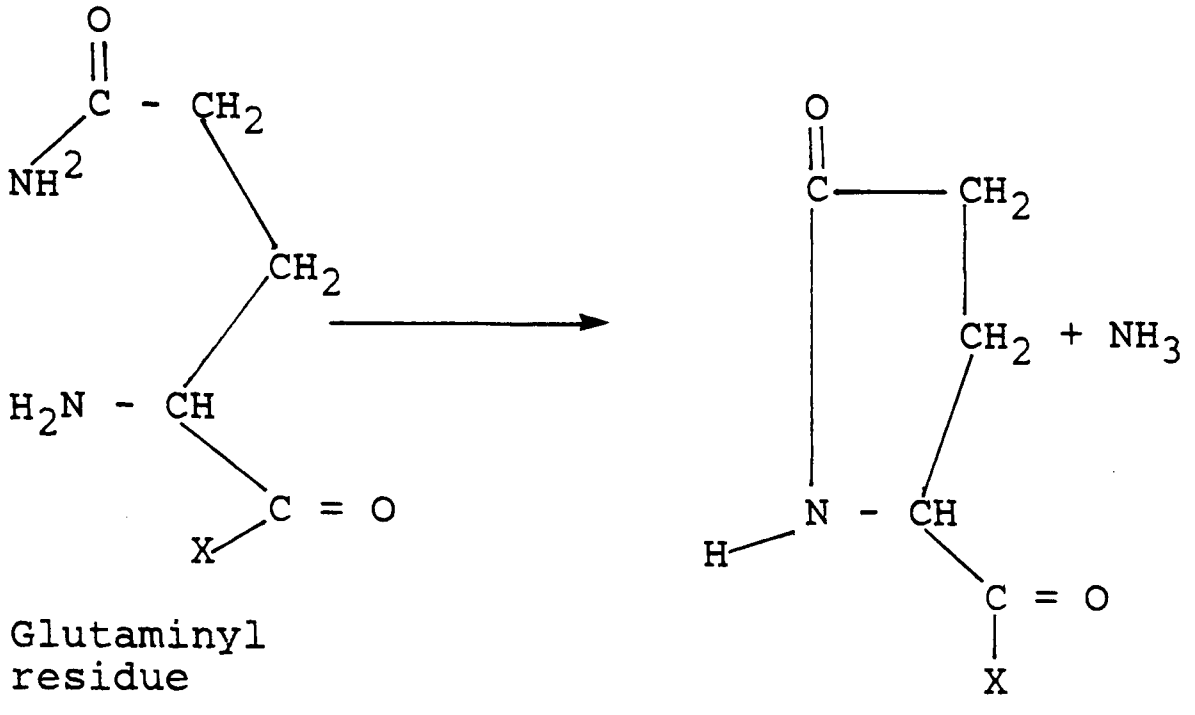
*The average hydrophobicity of these peptides was 1025 and 1200 (cal/res). This was less than the average hydrophobicity of most bitter peptides.

lysine residues (Kirimura et al., 1969). Valine, leucine, isoleucine, proline and tyrosine were found to occur most frequently as terminal amino acids (Schallnatus & Behnke, 1975a,b). Proline was found to be the most frequent amino acid in the bitter peptide from casein hydrolysate and bitter cheese (Gordon & Speck, 1965). Three bitter peptides from tryptic digest of casein were identified by Matoba et al. (1969), all of them had phenylalanine and proline in common.

Bitter peptides isolated from cheese were found to contain relatively large amounts of aliphatic, acidic and hydroxy amino acids; the ratio of aliphatic to acidic amino acids was found to be 0.8-1.3 (Edwards & Kosikowski, 1981).

Thirty-five bitter peptides isolated from casein hydrolysate and bitter cheese showed similarities in chromatographic behaviour, molecular weight and solubility (Schalinatus & Behnke, 1975b). On the other hand, Edwards & Kosikowksi (1981) isolated bitter peptides from Cheddar cheese and found that these peptides differed in some properties such as molecular weight, migration on silica gel and relative amino acid composition.

Infrared studies of bitter peptides derived from casein indicated the presence of cyclic structures such as azlacton, oxazolene and lactam (Tokita, 1969). Sullivan (1970) reported that the cyclic amide structure of lactam was that of pyrrolidonecarboxylic acid (PCA), a derivate formed by cylisation of N-terminal glutamine.



The bitter peptide disappeared when the cyclic peptide was hydrolysed to the non-cyclic form (Yamashita et al, 1969).

β-Casein is the main source of bitter peptides in milk and its products (Glegg et al., 1974; Visser et al., 1983). Visser et al. (1983) concluded that the C-terminal region of β-casein forms a principal source of bitter peptides in cheese. However, bitter peptides have been isolated from α_{S1}-casein (Hodges et al., 1972; Richardson & Creamer, 1973).

Lowrie & Lawrence (1972) proposed a model to account for the production of a bitter flavour in Cheddar cheese. They revealed that the starter streptococci were predominantly responsible for the development of the bitter peptides. The mechanism of the formation of the bitter peptide was proposed to be a three step process:

a) The breakdown of casein by rennet (chymosin), which produces a pool of high molecular weight peptides. These peptides are mostly non-bitter and they are precursors of the second step.

b) The hydrolysis of some of the high molecular weight peptides by proteases from Streptococcus spp leading to the formation of low molecular weight bitter peptides.

c) The bitter peptides may be further degraded by peptidases from streptococci forming non-bitter peptides and amino acids.

These workers concluded that the presence or absence of bitter flavour in Cheddar cheese depends upon manufacturing conditions rather than a single characteristic of the starter strains.

1.2.5 Effect of lipases secreted by psychrotrophic bacteria on milk and dairy products

Lipase activity has been reported for most of the psychrotrophs isolated from raw milk and cottage cheese (Gelpi, 1963). Reddy et al. (1968, 1969) found that P. fragi hydrolysed milk fat at 7°C within 6-9 d and liberated short chain fatty acids and that these short chain fatty acids (C4-C10) may be responsible for the production of fruity flavour in milk and cottage cheese. They concluded that short chain fatty acids served as the substrate for further esterification resulting in development of off-flavours in milk and dairy products. When milks were inoculated with psychrotrophic bacteria and incubated at 2-10°C for up to 48 h, the amounts of free fatty acids in all

samples were increased (Jensen & Hansen, 1974). The off-flavour in cheese was mainly due to butyric acid but a soapy taste caused by high molecular weight fatty acids, particularly dodecanoic and decanoic, was detected (Law et al., 1976). Chapman et al. (1976) inoculated raw milk with lipase producing psychrotropic bacteria of the genera Pseudomonas, Acinetobacter and Aeromonas. At a population number of 10^6 CFU/ml all these species produced enough lipase to cause rancid-flavours when milk was pasteurised and converted to cheese.

Lipolytic activity may cause deterioration of cheese manufactured from milk stored at 5°C. Lower yields, smoother textures and rancid flavours have been observed when compared to the control. The fatty acid analysis showed that the cheese made from stored milk had higher concentrations of long chain saturated fatty acids, un-saturated methyl ketones and volatile fatty acids (Dumont et al., 1977). Law et al. (1979b) working with P. fluorescens have shown that the lipase was an extracellular enzyme.

Lipases from psychrotrophs were found to have broad specificity, but the activity decreased as the chain length of fatty acids increased (Landaas & Solberg, 1978). P. fluorescens inoculated into milk produced the maximum amounts of free fatty acids at 5-10°C (Landaas & Solberg, 1978). Seventy-five percent of lipases from P. fluorescens and P. fragi grown in raw milk survived pasteurisation (Law et al., 1976). The thermostability of these lipases is probably due to a weak association between the lipase and

an unidentified stabilising factor (O'Donnell, 1978). The same workers found that cheese manufactured in the presence of P. fluorescens AR11, a prolific lipase producer, developed strong lipolytic rancidity after only two months' storage. Driessen & Stadhouders (1974) also found that the heat stable lipases of the psychrotrophs were capable of causing rancidity in UHT sterilised milk.

The effect of lipases on the other dairy products such as butter and cream has been investigated. Rancid flavour in raw cream was related to lipases secreted by Pseudomonas, Alcaligenes, Acinetobacter and Aeromonas (Jackson, 1978). Although butter is not a suitable medium for growth of bacteria because of the low content of water and the presence of salt , Pseudomonas species can grow and cause rancid flavour (Thomas, 1971). Eighty percent of the pseudomonas lipase activity was found in cream and subsequently in butter; the butter became rancid after two days at 5°C (Kishonti & Sjostrum, 1970).

1.2.6 Effect of phospholipases secreted by psychrotrophic bacteria on milk and dairy products

Phospholipases secreted by psychrotrophic bacteria may be important in the spoilage of milk. Fox et al. (1976) isolated 58 phospholipase producing strains from samples of fresh and spoiled homogenised milk. They found that 62% of the isolates were Pseudomonas species particularly P. fluorescens. These workers postulated that phospholipase (lecithinase) hydrolyses phosphorylcholine from the diglyceride moiety of lecithin thus increasing the susceptibility of the milk fat membrane to the action of lipases.

Chrispoe & Marshall (1976) reported that lipolysis of crude butter oil/soy lecithin emulsion was increased in the presence of phospholipase c from P. fluorescens.

In summary, extracellular lipases and phospholipases can affect the quality of heat-treated dairy products either by causing or enhancing deterioration of the flavour during storage. Hydrolytic rancidity caused by lipases is responsible for the appearance of rancid flavour in milk and its products.

1.3 Extracellular enzymes

1.3.1 Secretion of extracellular enzymes

A large number of bacteria secrete extracellular proteins into the external medium (Pugsley & Schwartz, 1985). Many of these proteins are enzymes which degrade large polymers (starch, cellulose, pectins, proteins, lipids and nucleic acids) to produce smaller molecules which can then be transported and metabolised (Glenn, 1976). Both Gram-positive and Gram-negative bacteria secrete proteins into the medium. The term extracellular enzyme was defined by Pollock in 1962 as "the protein which exists in the medium around the cell, having originated from the cell without any alteration to cell structure greater than the maximum compatible with the cell's process of growth and reproduction".

1.3.2 Models for the regulation of extracellular protein synthesis

There are two models proposed for the regulation of

the production of extracellular protein.

The first model was proposed by Coleman et al. (1975) based on the results obtained from the production of extracellular protein by Bacillus amyloliquefaciens. The pattern of production of extracellular enzymes predicted by this model has been observed for the secretion of lipase and protease by P. fluorescens (Roy, 1979; Juan & Cazzulo, 1976). It was proposed that during the logarithmic growth phase of a bacterium, the ribonucleotide pool is maintained at a constant level by feedback inhibition and repression. This nucleotide pool is utilised by three separate groups of RNA molecules: cell-protein mRNA, non-translatable RNA (tRNA + rRNA) and extracellular protein mRNA. The extracellular protein mRNA is considered to compete unfavourably with the other two forms of RNA. The cell protein mRNA is inhibited at the end of the logarithmic phase of growth. This leads to an increase in production of extracellular protein mRNA.

One disadvantage of this model is that it cannot explain the pool of extracellular protein specific mRNA observed in P. maltopohilia (Boethling, 1983), P. lemoignei (Stinson & Merrick, 1974), V. alginolyticus (Reid et al., 1980) and Bacillus spp (O'Connor et al., 1978; Priest, 1977). The explanation put forward by Coleman et al. (1975), however, was that this mRNA is normal with respect to quantity and lifetime. In a recent review, Priest (1983) suggested that the availability of RNA precursors is not a controlling factor for extracellular enzyme synthesis.

The second model was formulated by Harder (1979) and was proposed to explain the production of extracellular enzymes by Cytophaga flevensis and Vibrio SA1. Harder proposed that the regulation of extracellular enzyme synthesis is based on induction, end-product repression and/or catabolite repression. In this model it is summarised that when microorganisms grow in the absence of inducer, extracellular enzymes are produced at a very low level. In the presence of inducers these enzymes degrade exogenous substrates liberating low molecular weight inducing compounds. These compounds enter the cell and the organism obtains a signal indicating the presence of a potentially useful biopolymer. Under appropriate nutrient limitation, namely when the growth is limited by the availability of carbon for growth and energy production, extracellular enzyme synthesis is induced but may also be sensitive to negative feedback inhibition by the end products. Extracellular protease production by B. licheniformis fits in well with Harder's model (Wouters & Buysman, 1977). This enzyme was produced only when the inducer was present, irrespective of growth rate under nitrogen limitation. On the other hand, the production of alkaline phosphatase by the same organism was produced only under phosphorus limitation. Fairbairn & Law (1986a) concluded that there was good evidence to support Harder's model for the regulation of extracellular proteases of Gram-negative bacteria.

1.3.3 Mechanism of secretion of extracellular protein

Randall & Hardy (1984) distinguished four steps in the stage of secretion:

a) Interaction of the protein with the membrane;

b) Transfer of the secreted protein through the lipid bilayer;

c) Proteolytic removal of the leader sequence; and

d) The release of the matured protein from the membrane.

Pugsley & Schwartz (1985), on the other hand, conceive of the process of secretion as consisting of two steps, namely:

a) Export, which means the translocation to an extracytoplasmic compartment of the cell (including the plasma membrane), and

b) Secretion, which is a special case of protein export where the final destination is the outside of the cell.

Three models have been proposed to explain the mechanism of secretion of the extracellular protein. Based on the data obtained from eukaryotic cells, the signal hypothesis (Figure 1.3.1) was proposed. In this model the signal peptide emerges from the ribosome and interacts with a signal peptide recognition particle which is free in the cytosol. This interaction blocks further elongation and directs the signal peptide to the "docking protein" in the rough endoplasmic reticulum. A proteolytic enzyme "leader peptidase" located on the lumineal side of the membrane then removes the signal peptide (Blobel & Sabatini, 1971; Meyer, 1982; Meyer et al., 1982; Walter & Blobel, 1981a,b). Signal peptides are also found in exported

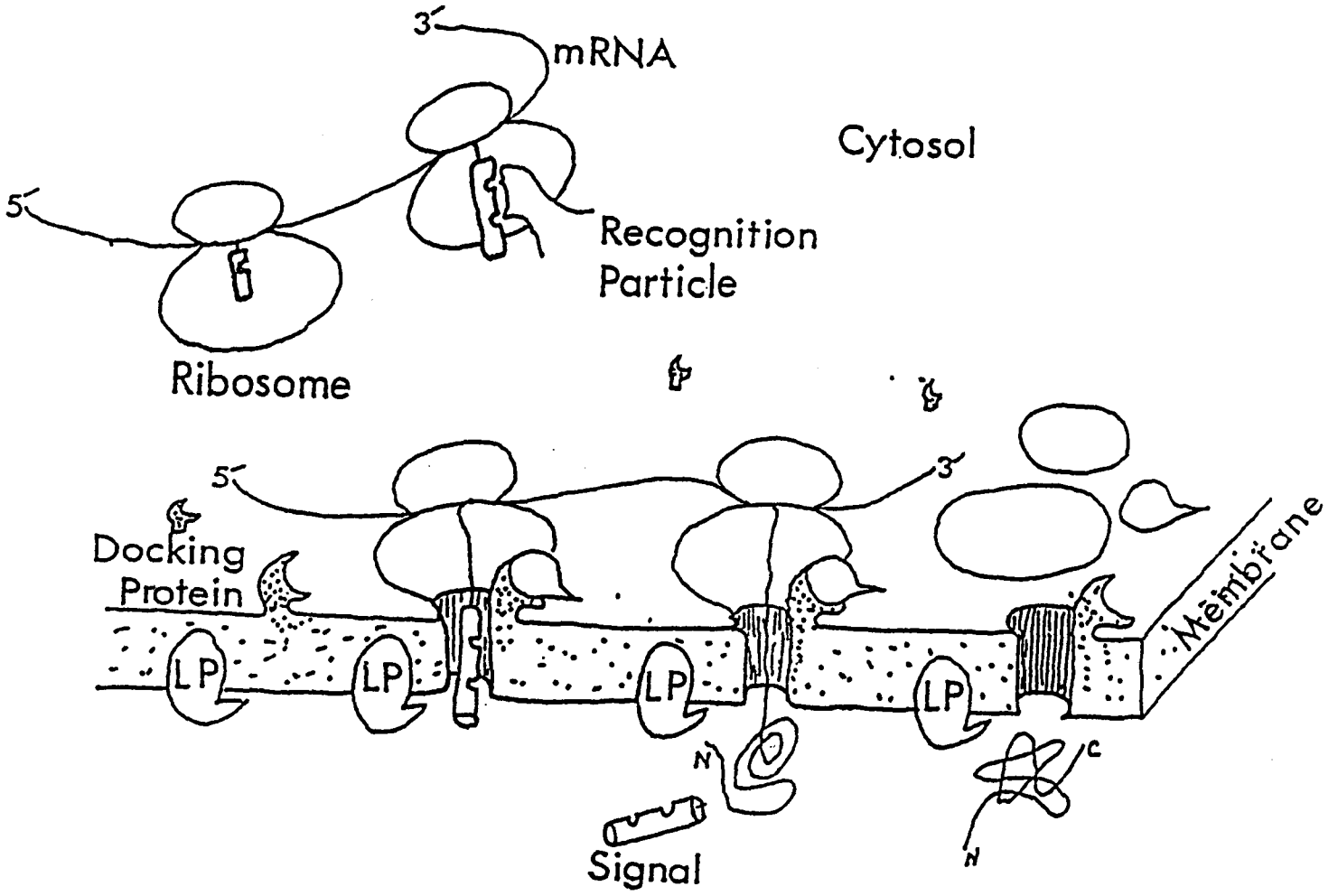


Figure 1.3.1 Signal peptide model: secretion of a protein through the rough endoplasmic reticulum (taken from Randall & Hardy, 1984)

LP = leader peptidase

proteins of prokaryotes, but it is not clear whether the mechanism of recognition and insertion of the peptide into the plasma membrane is the same as in eukaryotes (Müller et al., 1980; Crowelsmith & Gamen, 1982; Watson, 1984).

The second model, the membrane triggered folding hypothesis (Figure 1.3.2), was proposed by Wickner (1979; 1980) during the investigation of the infection of E. coli by the filamentous phage M13. This phage inserts its coat protein into the cytoplasmic membrane of the infected cell. Subsequent studies on the bacteriophage M13 (Ito et al., 1980; Date et al., 1980), indicated that the coat protein was synthesised as a precursor free in the cytoplasm and then inserted into the membrane only after its synthesis was complete. In this model the role of the aminoterminal leader sequence is not to bring the nascent polypeptide to the membrane, but to give conformation to the precursor that renders it soluble. A change in the conformation is caused by the interaction of the precursor with the membrane, resulting in association with the cytoplasmic side of the membrane. Proton motive force drives insertion across the lipid biolayer. This model has been criticised because coat proteins are not normal E. coli products and are not large enough for the ribosome to become membrane associated (Pugsley & Schwartz, 1985).

A third model was proposed by Randall & Hardy (1984). It came as a result of an investigation into the export of E. coli proteins. This can be described as a combination of the two models outlined above (Figure 1.3.3). After recognition and association with the membrane, the

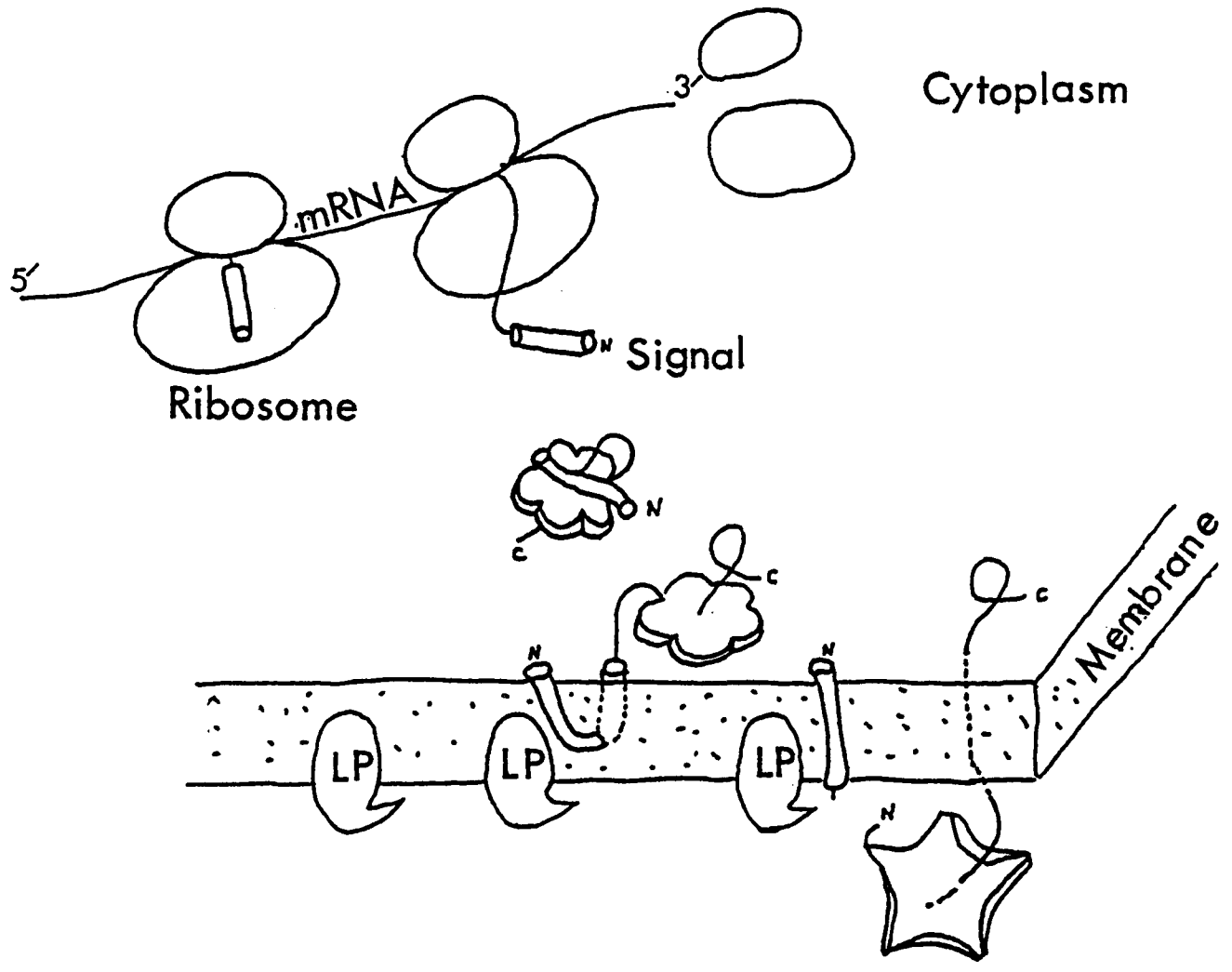


Figure 1.3.2 Membrane-triggered folding model: insertion of the coat protein of phage M13 into E. coli cytoplasmic membrane (taken from Randall & Hardy, 1984)

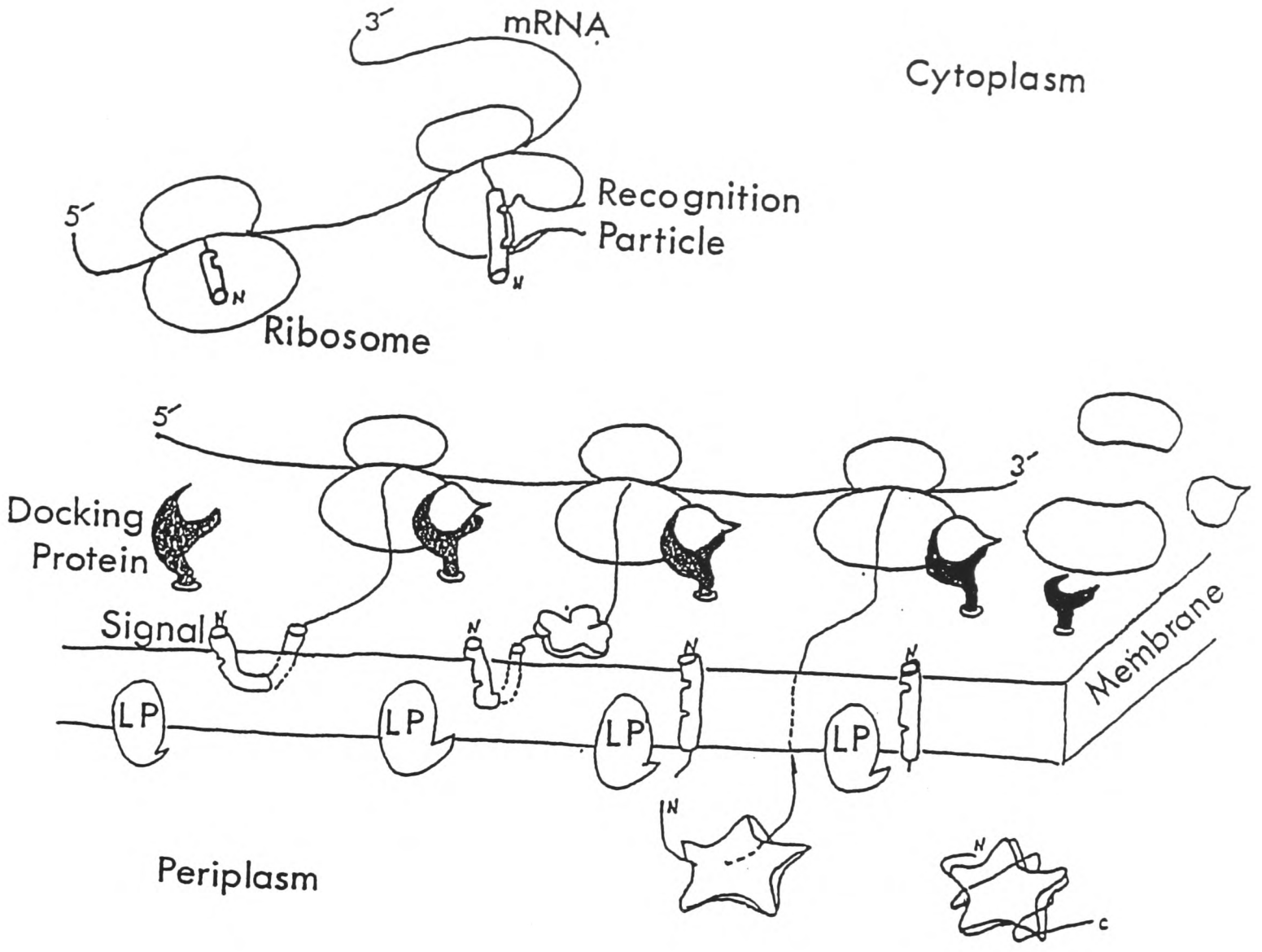


Figure 1.3.3 Working model incorporating signal hypothesis and membrane triggered folding model: transference of a soluble protein through the cytoplasmic membrane of E. coli (taken from Randall & Hardy, 1984)

polypeptide is elongated at the membrane. The mechanism of translocation is not precisely known yet. However, it seems to be more similar in nature to the membrane-triggered folding model than to the signal peptide model. From the studies by Josefsson & Randall (1981), Randall et al. (1978) and Varnee et al. (1978) on maltose-binding proteins, arabinose-binding proteins and alkaline phosphatase, it was revealed that translocation was independent of elongation. To find out whether there is a difference between the mechanism of secretion of protein in eukaryotic and prokaryotic cells, Talmadge et al. (1980) cloned eukaryotic proteins into E. coli and the proteins were secreted when the gene for a prokaryotic β-lactamase was introduced into either Saccharomyces cerevisiae or Xenopus oocytes, the bacterial plasmid (β-lactamase) was correctly processed (Roggenkamp et al., 1981; Wiedman et al., 1984).

It would seem no fundamental difference in the mechanism of secretion between eukaryotic and prokaryotic cells can be found, therefore the basis of secretion is fundamentally the same.

1.3.4 Regulation of extracellular protease biosynthesis

The action of extracellular proteolytic enzymes on proteins results in the release of peptides and amino acids. These products can be responsible for induction or repression of further synthesis of such proteases. Mechanisms of amino acid and peptide uptake are, therefore, relevant to the discussion of the regulation of protease biosynthesis.

1.3.5 Bacterial transport of amino acids

Transport systems in prokaryotes can be classified into three major groups: passive diffusion, facilitated diffusion and active transport. Passive and facilitated diffusion systems do not require energy input, whereas active transport requires an energy source (Antonucci & Oxender, 1986). Anraku (1980) used the term "active transport systems" to describe the uptake of amino acids from the environment by bacteria. These systems involve permeases which are located on the inner membrane of the cell of Gram-negative bacteria. The amino acids have no difficulty crossing the outer cell wall and reaching the permeases because of their small size. In E. coli K12, there are twelve kinetically defined transport systems for groups of structurally related amino acids (Table 1.3.1). Anraku (1980) pointed out that there was a similarity between the transport systems of E. coli and a wide variety of other bacteria. Two types of permeases were observed on studying the effect of osmotic shock on various transport activities in E. coli ML308-225. These types are:

a) The shock-sensitive systems; and

b) The shock-resistant systems.

Shock-sensitive systems are associated with periplasmic binding proteins and are absent in isolated membrane vesicles, eg those concerned with the uptake of glutamic acid and the branched chain amino acids. In these systems the role of the periplasmic binding protein components is not to translocate the substrate (amino acid) across the cytoplasmic membrane, but to increase the availability of

Table 1.3.1 Transport systems for amino acids in E. coli K12 (from Anraku, 1980).

Transport system		Substrate	Kt (μM)	Reference
1.	Glycine-alanine	Glycine	2.8-4.8	
		Alanine	27	Robbins & Oxender (1973)
1a.	Alanine	Alanine	2	
2.	Theronine-serine	Theronine	2	Templeton & Saveageau (1974)
2a.	Theronine	Theronine	0.39	Robbins & Oxender (1973)
3.	Leucine-isoleucine-valine			
	LIV-1	Isoleucine	0.40	
	LIV-2	Isoleucine	6	Yamato et al. (1975)
	LIV-3	Isoleucine	6	
4.	Phenylalanine-tyrosine-tryptophan	Phenylalanine	0.47	
		Tyrosine	0.57	
		Tryptophan	0.40	
4a.	Phenylalanine	Phenylalanine	2.0	Brown (1970)
4b.	Tyrosine	Tyrosine	2.2	
4c.	Tryptophan	Tryptophan	3.0	
5.	Methionine-1	Methionine	0.075	Kadner (1974)
	Methionine-2	Methionine	40	
6.	Proline	Proline	0.44 and 40	Morikawa et al. (1974)

Table 1.3.1 (cont'd)

Transport system	Substrate	Kt (μM)	Reference
7. Lysine-arginine-ornithine[g]	Lysine	0.5	Rosen (1971)
	Ornithine	1.4	
7a. Lysine	Lysine	10	Rosen (1971)
7b. Arginine	Arginine	0.026	
8. Cystine-1	Cystine	0.3	Berger & Heppel (1972)
Cystine-2	Cystine	0.02	
9. Asparagine-1	Asparagine	3.5	Willis & Woolfolk (1975)
Asparagine-2	Asparagine	80	
10. Glutamine-1	Glutamine	0.15	Weiner et al (1971)
Glutamine-2	Glutamine	2	
11. Aspartate-1	Aspartate	3.7	Kay & Kornberg (1971)
Aspartate-2	Aspartate	39	
12. Glutamate	Glutamate	0.7	Willis & Furlong (1975)
	Glutamate	10	

the substrate to the membrane carrier. Shock-sensitive systems require ATP, or a metabolite derived from ATP as a direct source of energy. On the other hand, shock-resistant systems are associated with carrier proteins which are not released by osmotic shock and which are active in membrane vesicles, eg transport systems for proline, phenylalanine, glycine, serine and cysteine. These systems are dependent upon gradients of ions and charge. These gradients are derived during either respiration or hydrolysis of ATP. Shock-resistant systems are proton-motive force dependent and may involve co-transport of protons or sodium ions. Both systems may be involved in particular amino acid transport, eg leucine.

Antonucci & Oxender (1986) revealed that the transport of branched-chain amino acids in E. coli is carried out by multiple systems. Three major systems have been identified. The LIV-I system transports L-leucine, L-isoleucine and L-valine; it also transports theronine and alanine, but with a lower affinity (Rahmanian et al., 1973; Anderson & Oxender, 1978). The second system LIV-II also transports the three branched-chain amino acids but has a much lower affinity than does the LIV-I (Anderson & Oxender, 1978; Antonucci & Oxender, 1986). The third transport system is called LS or leucine-specific transport system. It has a very high affinity for L-leucine and will also accept D-leucine but with a lower affinity (Wood, 1975; Anderson & Oxender, 1978).

Clarke & Ornston (1975) have summarised the information available about the amino acid transport systems in

Pseudomonas species. P. aeruginosa has stable constitutive permeases with high affinity (Km values were in the range 10^{-7} to 10^{-6} M) for 18 of the common amino acids. However, the basic amino acid permease of P. putida and the proline permease of P. aeruginosa are induced by their substrates and repressed by glucose. The regulation of amino acid transport and amino acid biosynthesis is controlled to meet the varying requirements of the cell (Clarke & Ornston, 1975; Oxender et al., 1980). The size and composition of the internal amino acid pools depends on the growth rate and environment of the cell (Fairbairn & Law, 1986a).

1.3.6 Transport and utilisation of peptides

Many bacteria utilise peptides as a source of amino acids. It has been well proven that peptides could enter the bacterial system and peptide cleavage would then occur intracellularly (Payne & Gilvang, 1968; Payne, 1968). Three transport systems for peptides in E. coli and S. typhimurium have been reported (Hogarth & Higgins, 1983; Higgins et al., 1983). Two have been well characterised for both bacteria: these are the dipeptide permease (dpp) and the oligopeptide permease (opp) systems. For the third system a tripeptide permease has been identified in S. typhimurium and its structural locus has been cloned (Hogarth & Higgins, 1983). Dipeptide permeation can occur via a permease(s) specific for dipeptides(s) (Payne, 1968), whilst the oligopeptide permease can transport peptides containing more than two amino acids, eg tetralysine, trityrosine, trivaline, pentaglycine. The two systems differ in specificity: the peptide permease requires a

free terminal carboxyl group on the substrate while the oligopeptide permease does not and can also transport peptides containing an esterified terminal carboxylic group (Shankman et al., 1962).

It has been reported that P. putida has separate and single uptake systems for both dipeptides and oligopeptides. Both systems were constitutive (Cascieri & Mallette, 1976). After uptake, peptides are rapidly hydrolysed by a wide range of intracellular peptides. Peptidases of P. putida and P. maltophila like those of E. coli appear to be constitutive and intracellular (Cascieri & Mallette, 1976).

It has been concluded that the superior nutritional value of peptides arises solely from the simultaneous supply of several amino acids following peptidase cleavage (Kihara & Snell, 1960a,b). The energy required for the peptide uptake is almost the same as for an amino acid, so less energy is needed for each amino acid taken up via the peptide (Bovena & Konings, 1986).

1.3.7 Induction and repression of extracellular proteases

Extracellular enzymes may be inducible, partially inducible or constitutive depending on the type of organism and the enzyme secreted (Priest, 1983). Extracellular proteases have been shown to be controlled by induction, end-product repression and/or catabolite repression (Monod et al., 1963; Fairbairn & Law, 1986a).

1.3.8 Induction by proteins and peptides

Proteins and peptides with molecular weight of 1000-60 000 induce or stimulate the biosynthesis of extracellu-

lar proteases (McKellar, 1982; McKellar & Cholette, 1984). The production of extracellular protease by P. fluorescens and P. aeruginosa isolated from raw milk was found to be dependent on the presence of organic nitrogen in the growth medium (Juffs, 1976). The same requirement for organic nitrogen for a clinical strain of P. aeruginosa has also been reported (Jensen et al., 1980a). The addition of protein to glucose basal medium stimulated the biosynthesis of the protease of P. aeruginosa. Bovine serum albumin was found to be more effective than casein (Whooley et al., 1983). By contrast the plant pathogen P. lachrymans synthesised a constitutive protease, whose production was not significantly affected by peptone, gelatin, casein or lactalbumin (Keen & Williams, 1967). α_{S2}-Casein was found to be the most effective stimulant for the production of protease of P. fluorescens NCDO 2085. K-Casein, α_{S1} and β-casein stimulated the protease production to the same level (Fairbairn & Law, 1987).

1.3.9 Induction by amino acids

The effect of amino acids on the biosynthesis of the extracellular protease depends on the metabolic character of the microorganisms and the type of amino acid involved (Jensen et al., 1980a). They found that glutamic acid and glutamine were the most effective amino acid inducers of the protease secreted by a clinical strain of P. aeruginosa. Glutamine was also found to induce the protease of a raw milk isolate of P. fluorescens when supplied as a sole carbon and/or nitrogen source. D-alanine and DL-alanine were found to stimulate the secretion of the

protease by P. fluorescens in basal medium by 10- and 3-fold respectively, when supplied as a sole carbon source (Amrute & Corpe, 1978; Vilu et al., 1980). All the amino acids, apart from serine, which were utilised as a sole source of carbon induced protease production to various extents by P. fluorescens NCDO 2085 (Fairbairn & Law, 1987).

Amino acids have been studied as protease inducers for a wide range of microorganisms. Glutamic acid was found to stimulate the induction of Micrococcus frendenreichii protease (McDonald & Chambers, 1966). Secretion of Streptococcus liquefaciens protease was induced by arginine, whilst leucine was the most effective amino acid inducer of the protease secreted by Serratia marcescens (Braun & Schmitz, 1980).

1.3.10 End-product repression

While amino acids can act as protease inducers, they may also cause repression of protease secretion under some circumstances. Amino acids can serve either as end-product repressors or inducers of protease secretion (Merkal et al., 1964; McDonald & Chambers, 1966; McKellar, 1982). High concentrations of amino acids repressed the secretion of protease by Neurospora crassa (Cohen & Drucker, 1977). Himelbloom & Hassan (1986) found that cysteine inhibited protease production and growth of P. fluorescens in a sodium caseinate medium, but only protease production was inhibited in a fish-extract medium.

1.3.11 Catabolite repression

Glucose and easily metabolised carbon sources repres-

sed protease secretion in Pseudomonas species (Juffs et al., 1968; Boethling, 1975; Whooley et al., 1983), Proteus mirabilis (Bonato et al., 1982), Serratia species (Snomke & Hammel, 1979), Aeromonas hydrophila (O'Reilly & Day, 1983) and Vibrio species (Wiersma & Harder, 1979; Lang et al., 1981). The repression of inducible enzyme by glucose is known as the "glucose effect" (Epps & Gale, 1942). It was proposed that glucose and other easily utilisable carbon sources are metabolised rapidly producing high levels of catabolic intermediates which cause repression of the synthesis of inducible enzymes, giving the term "catabolite repression" (Magasanik, 1961).

Addition of glucose to cultures of E. coli dramatically reduced the intracellular level of cyclic adenosine monophosphate (cAMP) (Makman & Sutherland, 1965). It was later suggested that cAMP was needed to promote the biosynthesis of inducible enzymes and that the "glucose effect" was due to the reduction of cAMP concentrations in the cells (Perlman & Pastan, 1968). Exogenous cAMP relieved the glucose effect and the role of cAMP in mediation of catabolite repression was confirmed by analysis of adenylate cyclase mutants, deficient in the enzyme which synthesises cAMP (Perlman & Pastan, 1969). However, in Pseudomonas and Bacillus species, cAMP does not appear to mediate catabolite repression (Priest, 1977).

Transient repression and inducer exclusion are two other different inhibitory effects on the utilisation of nutrients caused by glucose. Transient repression like catabolite repression, delays the synthesis of inducible or

constitutive enzymes for up to one generation time of the organism. Thereafter, enzyme synthesis begins at a rate characteristic of growth on glucose. On the other hand, inducer exclusion acts on the entry of the inducers into the cells rather than on the expression of genes directly (Kornberg et al., 1980).

1.3.12 Effect of other nutritional factors on the secretion of the extracellular proteases

Carbon and nitrogen sources are the most important factors in the biosynthesis of the extracellular proteases in Pseudomonas species. In addition to carbon and nitrogen some other nutrients such as metal ions play roles in the enzyme biosynthesis. Calcium chloride in the growth medium increased the yield of a protease secreted by P. fluorescens (Amrute & Corpe, 1978; Fairbairn & Law, 1987). Divalent cations such as Fe^{2+}, Zn^{2+} and Mg^{2+} can affect either the production or the activity of proteases secreted by some Gram-positive and Gram-negative bacteria (Bjorn et al., 1979; Jensen et al., 1980a,b; Hanlon et al., 1982). Phosphate ions also affect the synthesis of proteases. McKellar & Cholette (1984) reported that 5 mM orthophosphate was required for the maximum production of the protease secreted by a raw milk strain of P. fluorescens.

Kessler & Safrin (1983) revealed that ammonium and sodium ions inhibited the biosynthesis of protease and other extracellular proteins by P. aeruginosa. They suggested that reports about the inhibition by ammonium ions represented a non specific effect, most likely related to the high ionic strength of the medium.

1.3.13 Effect of the physical environment on the biosynthesis of proteases

Production of extracellular proteases is also influenced by the physical environment, eg temperature, aeration, pH and dissolved oxygen tension. For example, 20° and 30°C were the respective temperatures for the maximum production of protease by P. fluorescens and P. aeruginosa isolated from raw milk. Temperatures above the optimum temperature rapidly reduced the production of the enzyme although good growth was observed (Juffs, 1976). In pyschrotrophic bacteria, growth at low temperatures resulted in higher protease per unit dry weight than at optimum growth temperatures. It was noted that extracellular enzyme synthesis by P. fluorescens was reduced at temperatures near the maximum growth temperature. Sensitivity of enzyme synthesis to elevated temperature was also a property shared by marine psychrotrophs (O'Reilly & Day, 1983) and microorganisms associated with animal hides (Hare et al., 1981). Production of a protease secreted by Vibrio alginolyticus was inhibited by increasing the temperature from 30 to 37°C (Hare et al., 1981).

Protease secretion also depends upon the pH of the medium. Fairbairn & Law (1987) found maximum protease production occurred at pH 6.8 while only a relatively small amount of the enzyme was produced below pH 6.7 in P. fluorescens NCDO 2085.

Culture aeration was found to affect protease production. Keen & Williams (1967) reported that culture aeration of a plant pathogenic strain of P. lachrymans

increased the production of the protease. In contrast, Fairbairn & Law (1987) in a comparison of agitated and static cultures found that 30% less protease was produced per dry weight with agitation when compared to static culture.

Rowe & Gilmour (1982) reported that there was a drop in oxygen tension of the two strains of P. fluorescens grown in a simulated milk medium at 7°C for 10 d in a fermenter. This drop in oxygen tension was closely followed by enzyme production and this occurred when both strains reached the end of the logarithmic phase and the beginning of stationary phase. Brandt & Ledford (1982) investigated the effects of dissolved O_2 tension at 3°C and 9°C on the growth of Pseudomonas species from raw milk. They found that decreasing the temperature from 9°C to 3°C had a greater effect on reducing the growth rate than a decrease in oxygen tension from 9-12 to 1-3 ppm.

Nitrogen flushing or lack of oxygen was found to prevent the accumulation of proteolytic enzymes from psychrotrophic bacteria in raw milk (Murray et al., 1983). These researchers found that flushing raw milk, 1L with 100 ml nitrogen per minute at 4°C led to a longer lag phase and slower growth rate compared to the control. They also reported extensive proteolytic activity and degradation of β-casein in control milk but no detectable casein breakdown in nitrogen flushed milk.

1.3.14 Effect of phase of growth on extracellular protease production

The pattern of protease synthesis during batch growth

was investigated. McKellar (1982) reported that protease production by P. fluorescens strain 32A was observed in the late logarithmic and early stationary phase at both 20°C and 5°C. The same pattern was found by Juan & Cazzulo (1976) using P. fluorescens and by Keen & Williams (1967) with P. lachrymans. Production of extracellular protease by Pseudomonas species grown with protein as the sole source of carbon and nitrogen was found to be during the early exponential growth phase and continued throughout the growth cycle (Whooley et al., 1983). The protease of P. fluorescens NCDO 2085 was secreted in the exponential phase during growth on protein and in early stationary during growth on amino acids (Fairbairn & Law, 1987). Production of the extracellular protein on media with amino acids, or simple carbon sources begins in the late exponential growth phase and proceeds into the stationary phase. This pattern of production is a common feature of many bacteria secreting extracellular enzymes (Whooley et al., 1983).

In conclusion, the production of the extracellular protease is thought to be regulated by both induction and repression. Protein and specific amino acids induce the enzyme, whilst glucose and easily metabolised carbon sources are repressors. Ammonium ions and some amino acids also repress the production of protease. Other factors such as calcium and some other mineral nutrients affect the production of enzyme. Physical conditions such as temperature, pH and oxygen tension influence the production of the enzyme but to a lesser extent. Extracellular protein is secreted in the exponential phase during growth on protein

and in early stationary phase during growth on simple substrates.

1.4 Classification and properties of proteases

1.4.1 Classification

In 1960 Hartley proposed a general classification for proteolytic enzymes, dividing them into four groups according to their mechanisms of reaction. The groups are serine, sulphydryl (thiol), metallo and acid (carboxyl) proteases. These groups were further classified for microbial enzymes according to their side chain specificity (Morihara, 1974). For plant proteases Hartley's classification was modified by Ryan & Walker-Simons (1981) to give a total of six mechanistic sets (Table 1.4.1).

The four groups of proteases, as defined by Morihara (1974), are discussed in the following sections.

1.4.1.1 Serine proteases

This group includes the mammalian enzymes trypsin, chymotrypsin, elastase, thrombin, plasmin and the microbial enzyme subtilisin. All of these proteases have neutral or alkaline pH optima. The serine protease is so called because a serine residue is involved in the catalytic mechanism. They are irreversibly esterified and inhibited by organophosphorus reagents such as diisopropyl phosphonofluoridate, DFP (Hartley, 1960). The inhibition by DFP confirms that serine is in the active site of this group of proteases. Trypsin and chymotrypsin were the first enzymes to be crystallized, their three-dimensional structure was

Table 1.4.1 Classification of plant proteases according to Ryan & Walker-Simmons (1981)

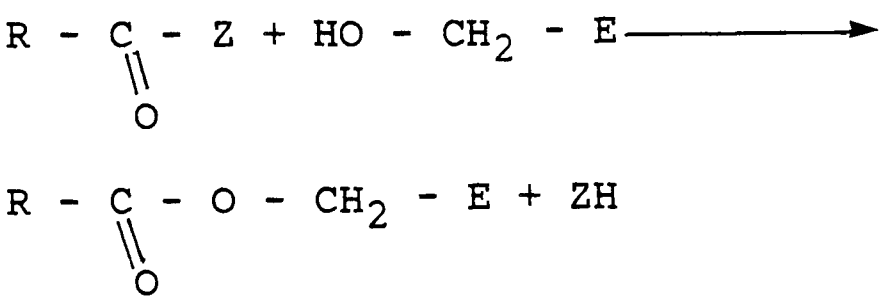
Group	Mechanistic set	Optimum pH	Example	Inhibitor
Sulphydryl endopeptidase	Cy-SH	5-7	Papain, ficin	IAA, PCMB
Acid endopeptidase	Acid	2-5	Pepsin, chymosin	Pepstatin
Serine endopeptidase	Serine	8	Trypsin, subtilisin	DIFP, PMSF
Serine exopeptidase	Serine	5-6	Carboxypeptidase C and Y	$HgCl_2$
Metallo-endopeptidase	Zn and Ca	7-9	Thermolysin	EDTA
Metallo-exopeptidase	Zn	7-9	Carboxypeptidase A and B	EDTA

IAA = iodacetic acid; PCMB = P-chloromericuribenzoate; DIFP = diisopropylfluorophosphate; PMSF = phenylmethane sulfonyl fluoride; EDTA = ethylenediaminetetracetic acid

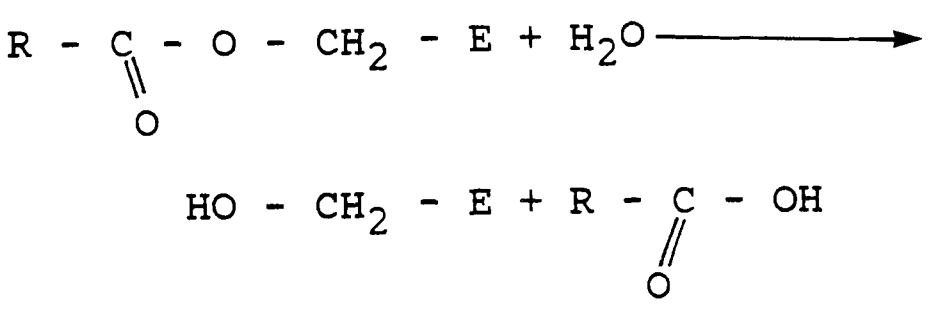
studied and the amino acid sequence determined (Whitaker, 1974).

With the exception of subtilisin, all serine proteases are secreted as inactive zymogens which are converted to active forms by specific proteolytic cleavages (Hartley, 1960; Whitaker, 1974). X-ray studies by Freer et al. (1970) and Wright (1973) have shown that substrate binding capability is a key distinction between active and inactive forms of the enzyme. Hydrolysis by chymotrypsin is a result of acylation and deacylation of Ser-195, which lies deep in a substrate binding pocket amongst His-57 and Asp-102 (Bender & Killheffer, 1973; Whitaker, 1974). Hydrolysis by chymotrypsin is shown in the diagram below.

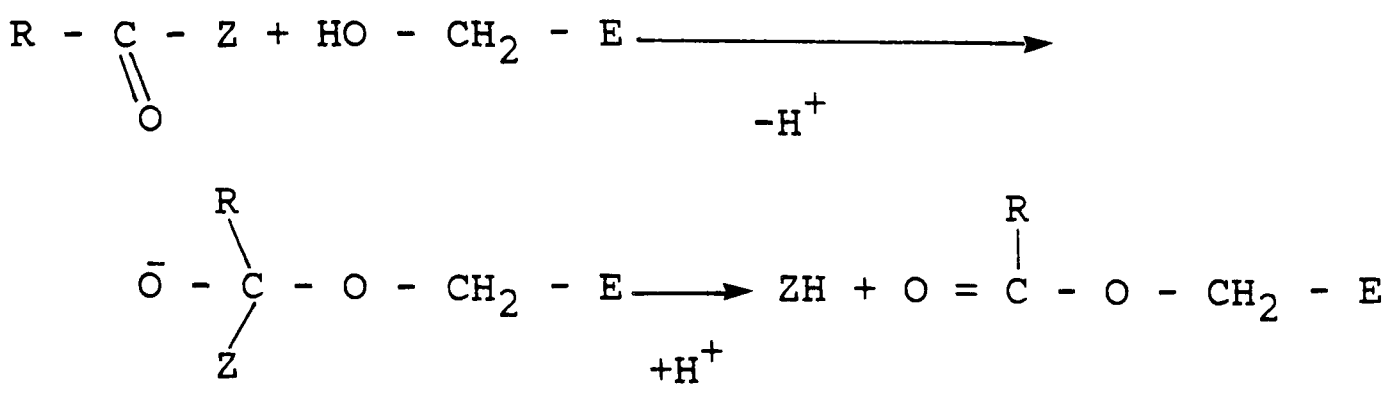
i) Acylation



ii) Deacylation



Two groups of workers suggested that the acylation reaction proceeds via a tetrahedral intermediate which is converted to the acyl enzyme by protonation of the leaving groups, Z. The diagram below shows the reaction (Bender & Killheffer, 1973).



Most of the work has been carried out using mammalian proteases and little information exists about the proteases of Gram-negative bacteria except E. coli protease I (Pacaud & Uriel, 1971). The microbial serine proteases are divided into at least four groups (Morihara, 1974). These groups are:

1.4.1.1.1 Trypsin-like proteases

Enzymes belonging to this group are produced by some species of Streptomyces such as S. griseus, S. fradial and S. erythreus (Trop & Birk, 1968; Morihara & Tsuzuki, 1968; Yoshida et al., 1971). The enzymes are specific for basic amino acid residues, and cleave on the carboxyl side of the splitting point. They are sensitive to specific trypsin inhibitors such as DFP, soybean trypsin inhibitor and tosyl-L-lysine chloromethyl ketone.

1.4.1.1.2 Alkaline serine proteases

A wide group of microorganisms are capable of producing proteases of this group, eg Bacillus spp, Arthrobacter spp, Streptomyces spp, Aspergillus spp, Penicillium spp and Saccharomyces spp (Markland & Smith, 1971; Matsubara & Feder, 1971). Subtilisin is an example of a bacterial alkaline serine protease. It is secreted by Bacillus subtilis and is economically one of the most important microbial proteases since it is used commercially as a

laundry detergent additive (Aunstrup, 1980). Alkaline serine proteases show specificity against aromatic or hydrophobic amino acid residues such as tyrosine, phenylalanine or leucine at the carboxyl side chain of the splitting point. They are inhibited by DFP, benzyloxycarbonyl-L-alanyl-L-phenylalanine chloromethyl ketone, benzyloxycarbonyl-L-alanyl-glycyl-L-phenylalamine chloromethyl ketone and potato trypsin inhibitor

1.4.1.1.3 Myxobacter α-lytic protease

Sporangium spp produce an example of this class of protease. This enzyme has strong bacteriolytic activity towards a number of soil bacteria (Morihara, 1974). It resembles pancreatic elastase in its specificity against oxidised insulin β-chain, and is specific for small aliphatic amino acid residues such as alanine at the carboxyl side of the splitting point. The only known inhibitor for this enzyme is DFP (Morihara, 1974).

1.4.1.1.4 Staphylococcal protease

This enzyme is only found in Staphylococcus aureus, is active over a wide pH range and only cleaves bonds that include, dicarboxylic amino acids present at the carboxyl side of the splitting point. It is sensitive to DFP (Morihara, 1974).

1.4.1.2 Sulphydryl proteases

These proteases are activated by reducing agents, eg HCN and are susceptible to sulphydyl blocking reagents such as PCMB. They are most active at neutral pH. They can be classified into at least two further sub-groups (Morihara,

1974). These groups are:

1. Clostripain protease.

2. Streptococcal protease.

1.4.1.2.1 Clostripain protease

Clostripain protease is produced by Clostridum histolyticum. It resembles bovine trypsin in being specific against basic amino acid residues at the carboxyl side of the splitting point. Clostripain is sensitive to tosyl-L-lysine chloromethyl ketone (a trypsin inhibitor) but DFP has little effect on it (Mitchell & Harrington, 1971). It possesses esterase, amidase and proteinase activity, but in all cases there is a very limited specificity directed primarily towards the carboxyl linkage of arginine (Morihara, 1974).

1.4.1.2.2 Streptococcal protease

Group A streptococci initially produce the enzyme as a zymogen which is converted autocatalytically into an active sulphydryl protease (Liu & Elliott, 1971). The enzyme is only inhibited by sulphydryl reagents such as PCMB. Results obtained using the oxidised insulin β-chain as a substrate showed that the chief requirement for hydrolysis is the presence of a bulky side chain on the amino acid adjacent (on the amino terminal side) to the residue contributing the carbonyl to the susceptible peptide bond (Trop & Birk, 1968; Wählby, 1968; Fruton, 1970). The streptococcal protease has a very broad specificity against either synthetic substrates or the oxidised β-chain (Morihara, 1974).

1.4.1.3 Metallo proteases

These enzymes are widely distributed in microorganisms. Metallo proteases are sensitive to chelating agents such as EDTA and o-phenanthroline. They may be classified into at least four groups:

1. Neutral proteases.
2. Alkaline proteases.
3. Myxobacter AL-I-protease I.
4. Myxobacter AL-I-protease II.

1.4.1.3.1 Neutral proteases

These proteases are most active at neutral pH. Thermolysin secreted by Bacillus thermoproteolyticus may be representative of this group. The amino acid sequence (comprising 316 amino acid residues) and the tertiary structure of this enzyme have been determined (Morihara, 1974). Experiments performed using thermolysin against different substrates such as oxidised β-chain insulin, tobacco mosaic virus and ferrodoxin showed that neutral enzymes are specific against hydrophobic or bulky amino residues such as leucine and phenylalanine at the amino side of the splitting point (Matsuhara et al., 1966; Matsuhara & Sasaki, 1968). Another example of a bacterial neutral metallo protease is subtilisin which is secreted by Bacillus subtilis. The hydrolysis of synthetic substrate Z-Gly-X-NH_2 at the Gly-X bond (X = various amino acid residues and Z = benzyloxycarbonyl) by subtilisin was studied by Feder (1967). Hydrolysis requires the presence of a dipeptide backbone containing alanine, valine, leucine or phenyl alanine on the amino terminal side of the cleavage

point. The highest rates of hydrolysis were observed with leucine.

Subtilisin can be classified as alkaline serine or metallo-neutral protease depending upon the strain which secretes it. When the enzyme is produced by Bacillus subtilis strain AJ3266, it was classified as alkaline serine protease (Morihara, 1974), whereas the strain used by Feder (1967) and Morihara et al. (1968) produced metallo-neutral protease.

The specificity of metallo-neutral protease was investigated by using two types of substrate - small molecular synthetic peptides and large molecular peptides or proteins. They generally showed specificity towards hydrophobic or bulky amino acid residues (Morihara, 1974).

1.4.1.3.2 Alkaline proteases

These enzymes are secreted by some Gram negative bacteria (Morihara, 1963; Morihara, 1974). They are most active against casein under mildly alkaline conditions (pH 7-9). These enzymes show broad specificity against large molecular peptides or proteins. Some differences have been observed between metal-chelator-sensitive neutral and alkaline proteases in their sensitivity to metal chelating agents. The former are usually sensitive to EDTA at concentrations less than 1 mM, whereas high concentrations are required to inhibit the latter (Morihara, 1963; McConn et al. 1964; Morihara, 1974).

P. aeruginosa and Serratia marcescens produce a collagenase-like protease that hydrolyses Z-gly-pro-gly-gly-pro-ala at the peptide bond gly-gly as clostridial

collagenase also does (Schoellman & Fisher, 1966; McQuade & Crewther, 1969). No regularity in specificity was observed in a study of P. aeruginosa alkaline protease with benzyloxycarbonyl-tripeptides as substrates, whilst the neutral protease cleaves peptide bonds containing bulky or hydrophobic bonds such as PHE-ALA or GLU-LEU (Morihara et al., 1973; Morihara, 1974).

Most extracellular proteases secreted by psychrotrophic Pseudomonas have been classed as neutral metalloproteases. They are specific for basic, bulky or hydrophobic amino acid residues at the amino side of the splitting bond (Fairbairn & Law, 1986a).

1.4.1.3.3 Myxobacter AL-I-protease I

Myxobacter strain AL-I produces two enzymes, protease I and protease II (Wingard et al., 1972). Protease I is a lytic enzyme capable of lysing the cell walls of Arthrobacter crystallopoietes. This enzyme has an affinity toward peptide bonds formed by at least one hydrophobic amino acid in a tetrapeptide, tetraglycine is hydrolysed with difficulty and triglycine and diglycine are not attacked at all.

1.4.1.3.4 Myxobacter AL-I-protease II

This protease in contrast to protease I does not lyse bacterial cell walls. It showed a high specificity for lysine residues at the amino side of the splitting point in the hydrolysis of substrates such as the oxidised insulin β-chain. Cytochrome c (horse heart) and lysozyme, small molecular peptides such as dilysine and trilysine were not hydrolysed (Wingard et al., 1972).

1.4.1.4 Acid proteases

The distinct characteristic of these acid proteases is their pH optima in the region of pH 2.0-5.0 and their insensitivity to the protease inhibitors DFP and PCMB. They are, however, sensitive to diazoketone compounds. These enzymes are not activated by reducing agents or metal ions (Sodek & Hofman, 1968). Acid proteases are widely distributed in moulds and yeast, but are rarely found in bacteria (Matsbura & Feder, 1971).

Microbial acid proteases have been divided according to their physiological characteristics into two groups: pepsin-like and rennin-like proteases. The former proteases are produced by Aspergillus, Rhizopus, Penicillium and Trametes spp. The latter group has been isolated from a variety of microorganisms including species of Mucor, Endothia, Bacillus and others. The substrate specificity of the enzymes towards the oxidised insulin β-chain is similar. They attack aromatic or bulky amino acid residues at both sides of the splitting point. The active centres of the acid proteases contain two aspartic acid side chains that share a common proton and a tyrosine hydroxyl. The presence of aspartic acid at the active site of the acid protease has led to them being referred to as aspartate proteases (Tang, 1979).

In conclusion, the specificity of proteases from Gram negative bacteria has received little attention and may be overshadowed by the economic significance of proteases from Bacillus spp. Studies on the proteases from Gram negative bacteria have been focussed primarily upon the detrimental

effects caused by their ubiquitous presence in the food and health industries. Examples of problems caused by these enzymes include degradation of milk and fish proteins, burned skin wounds (pneumonia) and sheep fleece disease caused by Pseudomonas spp (Adams et al. 1976; Gebre-Egziabher et al., 1980; Fox 1981, 1982; Venugopal et al., 1983; Shibl & Al-Sowaugh, 1980; London, 1984).

Understanding specificity of proteases from Gram negative bacteria will help to obtain the most desirable hydrolytic reactions. These enzymes may have a commercial use in the future because most of them are heat stable.

1.4.2 Properties of extracellular proteases from Pseudomonas species

Comparison of the physical properties of proteases secreted by Pseudomonas spp has shown similarities between these enzymes. In general the enzymes have molecular weights of 35 to 55 K daltons, are active in the range 30-45°C and pH 6-9, are inactivated by metal chelators, and are reactivated by divalent cations particularly calcium. One of the most notable characteristics is their heat stability; these proteases can withstand high temperature treatments and may cause problems in milk and its products (Section 1.2).

1.4.2.1 Heat stability

Adams et al. (1975) found that an extracellular protease of Pseudomonas MC60 was 4000 fold and 400 fold more heat stable than spores of Bacillus stearothermophilus and Clostridium sporogenes PA3679. Similar findings have been reported by other workers. The heat stability of some heat

resistant proteases from Pseudomonas spp is summarised in Table 1.4.2.1. All the proteases of Pseudomonas spp isolated from raw milk by Griffith et al. (1981) retained about 55-65% of their initial activity after exposure to 77°C for 17 s, and about 20-40% after treatment at 140°C for 5 s. Three proteases secreted by P. fluorescens AFT21 were studied for their heat stability in both phosphate buffer and synthetic milk ultrafiltrate (SMUF). Their D values at 140°C were 69, 54 and 80 s respectively (Stepaniak & Fox, 1985). Heating the protease secreted by P. fluorescens T16 at 50, 90 and 120°C resulted in a rapid initial loss of more than 50% of initial activity followed by a gradual inactivation which showed first order kinetics (Patel et al., 1983). Twenty-six percent of the initial activity was retained after heating the protease from P. fluorescens at 70°C for 10 min , but only 14% of its activity was lost after heating at 45°C for 30 min (Malik & Mathur, 1984). The heat stability of six proteases from six different strains of Pseudomonas fluorescens was studied ; five out of six of these enzymes were able to withstand ultra heat treatment with D-values at 140°C ranging from 2-300 s (Mitchell et al., 1986). Barach et al. (1976a) found that the half life of a protease of Pseudomonas spp MC60 at 149°C ranged from 2-7.5 s. Richardson & Te Whaiti (1978) studied seven enzymes for heat stability, four of them retained an average of 57% activity after being heated for 30 s and 26% activity after heating for 60 s at 149°C.

The different conditions used by various workers make the comparison of the reported heat stabilities between

Table 1.4.2.1 Thermostability of heat-stable proteases secreted by Pseudomonas species

Organism	Temperature (°C)	Time (s)	% Residual activity	Reference
Pseudomonas sp	77	7	55-65	Griffith et al. (1981)
	140	5	20-40	
Pseudomonas fluorescens NCDO 2085	74	17	40	Fairbairn & Law (1986b)
	140	4	70	
Pseudomonas fluorescens	70	600	26	Malik & Mathur (1984)
	45	1800	86	
Pseudomonas fluorescens MC60	149	7	50	Barach et al. (1976a)
Pseudomonas fluorescens	149	30	57	Richardson & Te Whaiti (1978)
	149	60	26	
	144	30	10	
Pseudomonas fluorescens AR11	150	8.5	50	Alichanidis & Andrews (1977)

different species difficult. Heat stability varies according to the medium in which the enzyme is heated. Proteases are more stable in the presence of skim milk, whey and casein than in buffer (Mayerhofer et al., 1973; Barach et al., 1976a; Fairbairn & Law, 1986a). Loss of heat resistance has been reported to be due to autolysis of proteases in buffer which would not occur in the presence of milk proteins (Mayerhofer 1973; Richardson, 1981; Birkeland et al, 1983). Barach et al. (1976a) reported that the heat stability of a protease of Pseudomonas spp in milk during UHT treatment was associated with its interaction with Ca^{2+}, which has been shown to stabilise many proteolytic enzymes (Feder et al., 1971). In the absence of Ca^{2+}, the heat stability of the protease of Pseudomonas MC60 in milk was markedly reduced (Barach et al., 1976a). Barach et al. (1976a) also found that Ca^{2+} and Zn^{2+} were required for the optimum activity of the enzyme, but only Ca^{2+} restored the protective effect of milk against high temperature inactivation. Pseudomonas proteases usually lack disulfide bridges and calcium can stabilise the protease resulting in increased heat resistance (Cogan, 1977; Stepaniak & Fox, 1983; Patel et al., 1986). It has been proposed that calcium could interact to stabilise the negatively charged protease structure (due to a high percent of acidic amino acids, eg aspartic and glutamic) (Morihara, 1964). It has also been suggested that the heat stability of such proteases is due to the structural flexibility as a result of the lack of cysteine and the interchange of divalent cations which allowed rapid and accurate renaturation when

the temperature was lowered again, rather than maintenance of native structure during heating at high temperatures (Barach & Adams, 1977).

The D-values of some of the reported heat stable enzymes are summarised in Table 1.4.2.2.

Although most extracellular Pseudomonas proteases are heat stable at UHT treatment temperatures, some of them have been reported to be unstable at 55°C. Barach et al. (1976b) found that 10% of the initial activity of eight proteases remained after heating at 55°C for 10 min. Similar results have been found by several authors (Dalaly & Abbo, 1982; McKellar & Cholette, 1983; Patel et al., 1983). However, Mitchell et al. (1986) found that low temperature inactivation (LTI, 55°C for 1 h) had little effect on the activity of four proteases isolated from different strains of P. fluorescens. Stepaniak & Fox (1983) reported that the three heat stable proteases they investigated underwent autolysis and were unstable at 55°C for 10 min.

The inactivation of proteases at 55°C appears to be due to autolysis (West et al., 1978; Barach et al., 1978; Stepaniak & Fox, 1983). Barach et al. (1978) investigated the mechanism of low temperature inactivation of Pseudomonas MC60, and suggested that this inactivation occurred in two stages. The first stage involved a conformational transition at 55°C, which modified the protein structure. As a result of this, there was reversible loss of catalytic activity and susceptibility of the enzyme to proteolysis by other enzyme molecules which were still active. The next

Table 1.4.2.2 D values of proteases secreted by *Pseudomonas* species

Organism	Test medium	Temp °C	D value min	Reference
Pseudomonas spp 21B	Skim milk	74 130	160 8.8	Kishonti (1975)
P. fluorescens MC60	Jenness-Koops buffer solutions	74 149	304 1.5	Adams *et al*. (1975)
P. fluorescens NCDO2085	0.05 M KH_2PO_4 pH 6.5	140 150	1.0 0.5	Alichanidis & Andrews (1977)
P. fluorescens AFT36	Synthetic milk ultrafiltrate pH 6.6	70 140	219 1.0	Stepaniak & Fox (1983)
P. fluorescens AFT21 protease I	Synthetic milk ultrafiltrate pH 6.6	70 140	149 1.15	Stepaniak & Fox (1985)
protease II		70 140	118 0.90	Stepaniak & Fox (1985)
protease III		70 140	239 1.33	Stepaniak & Fox (1985)
P. fluorescens NCDO2085	0.05 M KH_2PO_4 pH 6.5	74 140	1.6 1.0	Fairbairn & Law (1986b)
P. fluorescens OM2	Synthetic milk ultrafiltrate pH 6.6	74 140	41.0 3.5	Mitchell *et al*. (1986)

Table 1.4.2.2 (cont'd)

Organism	Test medium	Temp °C	D value min	Reference
OM41		74 140	157 5.0	Mitchell et al. (1986)
OM82		74 140	190 2.0	Mitchell et al. (1986)
OM186		74 140	38.5 0.53	Mitchell et al. (1986)
OM228		74 140	13.5 0.12	Mitchell et al. (1986)
OM1191		74 140	13.0 0.25	Mitchell et al. (1986)
OM1192		74 140	5.5 0.03	Mitchell et al. (1986)
P. fluorescens P1	Synthetic milk ultrafiltrate pH 6.6	150	0.8	Stepaniak et al. (1987)

stage involved aggregation of the thermal altered protease with casein micelles to form an enzyme-casein complex. Aggregation and autolysis in milk were prevented by high concentration of NaCl or urea. The protease secreted by P. fluorescens 5613 lost 70% of its activity after heating at 77°C for 17 s followed by 55°C for 1 h but when following the reverse process, the enzyme was completely inactivated. One application of this technique was carried out by West et al. (1978). They found that the shelf life of UHT milk increased 3-fold when LTI was used in addition to the UHT treatment. This treatment did not harmfully affect the flavour or the quality of the milk.

1.4.2.2 Amino acid composition of proteases

The amino acid composition of purified proteases from Pseudomonas species are significantly similar. Aspartic acid, glycine, alanine, serine and glutamic acid are the major amino acids in the protease structure, while methionine and cysteine are the least frequent amino acids. Table 1.4.2.3 summarises the amino acid composition of various proteases. The lack of cysteine, for disulfide bridge formation, is proposed to facilitate secretion of the enzyme into the surrounding medium (Payne, 1975; Law, 1980). The overall similarities of the amino acid composition of extracellular proteases may contribute to a common mechanism for synthesis and secretion of these enzymes in Gram-negative bacteria.

1.4.2.3 Molecular weight

The molecular weight of proteases secreted by Pseudomonas species is in the range of 35-50 K daltons. The

Table 1.4.2.3 Amino acid composition of extracellular proteases from Pseudomonas species

Reference	1	2	3	4	5
Amino acid (residues/mol)					
ASP	67	66	46	85	82
GLY	62	65	70	76	65
ALA	44	58	56	59	48
SER	47	42	26	43	36
VAL	26	25	24	40	21
THR	36	24	22	37	50
LEU	27	37	21	34	19
PHE	22	20	12	27	21
GLU	29	35	27	26	28
ILE	16	17	11	24	40
TYR	22	21	0	18	21
LYS	17	16	19	11	13
HIS	11	6	8	9	5
ARG	6	7	10	9	3
PRO	8	11	0	9	8
TRP	6	6	ND	4	4
MET	2	0	4	4	2
CYS	0	0	0	0	0

Table 1.4.2.3 (cont'd)

Reference	6	6	6	6	6	6
Amino acid (residues/mol)						
ASP	63	63	67	60	61	54
GLY	58	63	66	61	65	65
ALA	54	54	53	48	59	51
SER	27	34	34	41	35	26
VAL	26	30	28	27	25	31
THR	41	41	32	47	41	32
LEU	33	30	25	26	28	39
PHE	21	21	22	22	36	18
GLU	30	30	29	31	31	32
ILE	20	23	34	16	16	20
TYR	18	18	22	22	16	26
LYS	23	24	18	25	21	25
HIS	12	10	11	13	8	12
ARG	9	7	7	9	10	11
PRO	10	8	9	11	15	17
TRP	4	5	3	3	3	6
MET	0	2	2	1	0	0
CYS	0	0	0	0	0	0

1 - Richardson, 1982
2 - Morihara et al., 1964
3 - Malik & Mathur, 1984
4 - Noreau & Drapeau 1979
5 - Barach & Adams, 1977
6 - Mitchell et al., 1986

survey of Patel et al. (1983) and Fairbairn & Law (1986a) confirmed these figures. Only one protease was reported to have molecular weight of 23 K daltons (Mayerhofer et al., 1973).

1.4.2.4 Optimum temperature

The optimum temperature for proteolytic activity of proteases from various Pseudomonas species was found to be between 30 and 45°C. Table 1.4.2.4 shows a summary of the physicochemical properties of some extracellular proteases of Pseudomonas species. No activity was found at 5°C for P. putrefaciens protease (Van der Zant, 1957). However, Alichanidis & Andrews (1977) and Stepaniak et al. (1982b) have found that the enzyme of P. fluorescens possessed about 30% of its maximum activity at 4 and 7°C respectively. The proteolytic activity of the protease declined sharply above 50°C and no activity was detected at 55°C (Adams et al., 1975).

1.4.2.5 Optimum pH

The optimum pH for Pseudomonas species proteases varied with the strain and substrate on which the enzyme was assayed. Generally speaking, optimum pH was found to be neutral to slightly alkaline with appreciable activity between 6.0-9.0. Morihara (1963) found that a protease from P. aeruginosa was very unstable at pH 3.5 or above pH 11.0.

1.4.2.6 Activation energy

Proteases secreted by Pseudomonas spp appear to be well adapted for growth at low temperature. This is reflected in the low activation energy. The activation

Table 1.4.2.4 Physicochemical properties of some proteases of *Pseudomonas* species

Strain	*P. fluorescens* T25	*P. fluorescens* CDO 2085	*P. fluorescens* Protease OM2	*P. fluorescens* Protease OM411	*P. fragi* ATCC 4973	*P. fluorescens* 16	*P. fluorescens*
Reference	Jackman *et al.* (1983)	Fairbairn & Law (1986b)	Mitchell *et al.* (1986)	Mitchell *et al.* (1986)	Porzio & Pearson (1975)	Patel *et al.* (1983)	Yan *et al.* (1982)
Class	Metallo-neutral	Metallo	ND	ND	Metallo-neutral	Metallo-neutral	Metallo-neutral
Optimum temperature	ND	ND	45	44	40-45	37	
Optimum pH	6-7	ND	8.3	7.8	6.5-8.0	7.4	7
Molecular weight	43,000	40,200	47,275	47,784	45,000	37,800	44,000
Metal content	ND	Ca, Zn	Ca, Zn	Ca, Zn	Zn	ND	ND
Inhibitor	ND	EDTA, EGTA, o-phenanthroline	EDTA	EDTA	EDTA	EDTA	ND
Activator	Ca, Mn	ND	Ca, Zn	Ca, Zn Mn, Ca	Zn, Ca,	Ca, Mn	ND

Table 1.4.2.4 (cont'd)

Strain	*P. fluorescens* P26 protease	*P. fluorescens* R12	*P. fluorescens* B52	*P. fluorescens* MC60	*P. fluorescens* CDO 2085
Reference	Mayerhofer *et al.* (1973)	Juan & Cazzalo (1976)	Richardson (1981)	Barach *et al.* (1978)	Alichanidis & Andrews (1977)
Class	ND	Metallo	Metalloalkaline	Metalloneutral	Thio
Optimum temperature	21	30	45-50	45	35
Optimum pH	7.5	6.5-7.0	7.0	7.5	6.5
Molecular weight	23,000	37,000	46,900	48,000	38,400
Metal content	ND	Ca	Ca, Zn	Zn, Ca	ND
Inhibitor	ND	o-phenanthroline	EDTA	EDTA	IAA, Zn, Pb
Activator	ND	Co, Zn, Ca	Co, Zn, Cu, Ca	Ca, Zn	EDTA

energy required for the breakdown of casein by P. fluorescens T16 protease was low compared to trypsin. The activation energy was 3.2 K cal/mol and 12 K cal/mol respectively (Patel et al., 1983). Similar low activation energies were observed for seven proteases from Pseudomonas species (Jackman et al., 1983).

1.4.2.7 Carbohydrate content

Most of the reported studies on the properties of proteases from different Pseudomonas spp found no evidence for the presence of carbohydrate in the enzymes. However the protease from Pseudomonas 145 contained 10% hexose and 21% glucosamine and galactosamine (Makino et al., 1983). The main protease of P. aeruginosa strain 34362 contained 10% glucosamine (Jensen et al., 1980c). Mitchell et al. (1986) studied the carbohydrate content of six proteases of different strains of P. fluorescens, they all contained carbohydrate, ranging from 0.03-0.23%. Although the carbohydrate content is low, they classified these enzymes as glycoproteins.

1.4.2.8 Classification

The proteases of Pseudomonas species are classified as metallo-proteases. Most of the reported enzymes are either metallo-neutral or metallo-alkaline proteases. Metallo-proteases require divalent cations for activity and stability. Their apoenzymes are activated by Zn^{2+}, Ca^{2+} and cobalt. Zinc has been shown to be essential for the activity of a number of bacterial extracellular proteases (Pangburn et al., 1976). Calcium and zinc were the major metals present in the extracellular proteases from Pseudo-

monas fluorescens studied by Richardson (1981), Fairbairn & Law (1986b) and Mitchell et al. (1986).

1.4.2.9 Inhibitors

Chelating agents such as EDTA and o-phenathroline were found to inactivate metallo-proteases (Jackman et al., 1983; Fairbairn & Law, 1986b). Metallo-neutral proteases are more sensitive to EDTA than metallo-alkaline proteases. The former are usually sensitive to EDTA at concentrations less than 1 mM (McConn, 1964).

1.4.2.10 Substrate specificity

Limited information is available on the specificity of Gram-negative bacterial proteases. It was reported that neutral metallo-proteases have a preference for basic and bulky or hydrophobic amino acids at the amino side of the splitting bond (Morihara, 1974; Fairbairn & Law, 1986a). Pseudomonas aeruginosa alkaline protease showed no regularity in hydrolytic activity, while the neutral protease hydrolysed peptide bonds containing bulky or hydrophobic bonds (Morihara et al., 1973; Morihara, 1974). Mitchell et al. (1986) studied the specificity of eight proteases (using synthetic peptides), six from different strains of P. fluorescens and two from Serratia marcescens. They concluded that the proteases appeared to have a narrow specificity for low molecular weight peptide substrates, but showed a broad specificity towards large molecular weight peptide or protein substrates. The protease of P. fragi ATCC 4973 showed a different specificity in that it hydrolysed bonds involving small and hydrophilic residues (Noreau & Drapeau, 1979).

1.5 Control of psychrotrophic bacteria and their secretion of proteases in milk

1.5.1 Preventing contamination by psychrotrophs

Preventing contamination of milk by psychrotrophic bacteria is practically difficult and expensive (Speck & Adams, 1976). Thomas & Thomas (1973a) considered that the best possible way to minimise the number of psychrotrophs in milk was through good hygienic practice, in the pipelines of dairy plants, farm bulk tanks, transport tanks and dairy equipment in the plant. The number of Gram-negative bacteria was low on dairy equipment that was steam sterilised or cleaned with hot water detergent-hypochlorite solutions (Thomas, 1974b). Colony counts of psychrotrophs higher than 10^5 CFU/ml (determined at 7°C for 10 days) gave an indication of unsatisfactory production conditions, whilst less than 10^4 CFU/ml indicated satisfactory standards of hygiene for the handling of milk (Thomas & Thomas, 1973b).

1.5.2 Control of growth and metabolism by psychrotrophs

1.5.2.1 Refrigeration

Low temperature has been the primary method used to control bacterial growth in raw milk. Keeping milk at or below 4°C during storage extends the lag phase of the psychrotrophs and consequently the keeping quality of the milk. Refrigeration also reduces the activity of proteases secreted by these bacteria since most of these enzymes have their optimum temperature above 30°C (Law, 1979) and become less active below 5°C (Juffs, 1976).

Raw milk of good quality can be stored at 5°C for up

to 3 d without any effect on quality (Mabbitt, 1980; Stadhouders, 1982). However, keeping raw milk at temperatures lower than 4°C causes the caseins to dissociate and may affect the properties of the milk (Reimerdes, 1982).

1.5.2.2 Thermisation

In the Netherlands, dairy processors use a special heat treatment called "thermisation". In this process the incoming milk is heated at 64-68°C for 10-15 s immediately after its arrival, followed by storage below 7°C. Thermisation reduced the bacterial count and extended the lag period, probably due to thermal shocking (Zall et al, 1982; Fonden, 1982). Thermisation might induce germination of spores of Bacillus cereus, then killed by subsequent pasteurisation. On the other hand, the additional use of pasteurisers for thermisation would require more cleaning to prevent the build-up of Streptococcus thermophilus in the pasteuriser. After this process milk could be stored for extended periods without affecting the quality of the product.

The shelf life of cottage cheese manufactured from thermised milk was of the same quality or even better than control unheated milks (Dzurec & Zall, 1982). Thermisation does not have an effect on pH, whey protein or coagulation time after pasteurisation compared to control milks (Coghill et al., 1982). Although thermisation treatment has many advantages, cooling is economically cheaper than thermisation and does not need complex equipment.

1.5.2.3 Addition of lactic acid bacteria

Inhibition of psychrotrophic bacterial growth by the

addition of 0.5% (v/v) lactic acid bacterial cultures to refrigerated milks has been suggested by Price & Lee (1970) and Juffs & Babel (1975). The inhibitory effect of lactic acid-producing streptococci or lactobacilli was characterised by high acidity (lactic acid), production of H_2O_2, low redox potential in the milk and increased lag phase of psychrotrophs.

Lactic acid bacteria also produce bacterial inhibitors, eg the antibiotic acidophilin from Lactobacillus acidophilus, which inhibited the growth of psychrotrophs (Hosono et al., 1977). Another example is the heat stable amine produced by Streptococcus thermophilus (Pulusani et al., 1979).

It has been reported that cheese made from milk seeded with lactic acid bacteria in USA dairy plants was better than the control cheese and that the pasteurised milk had a longer shelf life (Honer, 1981). The production of lactic acid by lactic acid bacteria will be an advantage only if the milk is manufactured into cheese because the slightly lower pH shortens the coagulation time. On the other hand, the formation of lactic acid may be a problem if the milk is used for other dairy products. In addition, some off-flavours could be produced as a result of the growth of lactic acid bacteria (Speck & Adams, 1976).

1.5.2.4 Activation of inhibitory system in milk

It is well known that raw milk has bactericidal and bacteriostatic properties. These naturally occurring inhibitory systems in raw milk include complements and specific antibodies, lactoferrins, lysozymes, leukocytes

and lactoperoxidase (LP). The lactoperoxidase system previously known as "lactenin" is the most effective inhibitory system; it was first used to inhibit the growth and acid production of various streptococci (Oram & Reiter, 1966; Reiter & Oram, 1967). This system involves three components, lactoperoxidase, thiocyanate and hydrogen peroxidase. Lactoperoxidase and thiocyanate (production depends on the feed given to the cows) occur naturally in milk at concentrations adequate for inhibition. The limiting factor is H_2O_2, which is not known to occur naturally in milk. However, production can be initiated by the addition of glucose and glucose oxidase to the milk. The inhibitory effect of this system is due to the inhibition of glycolytic enzymes by an intermediate oxidation product such as $S(CN)_2$ or HOSCN from the LP-catalysed oxidation of thiocyanate by hydrogen peroxide (Bjorck et al., 1975).

Catalase-negative bacteria such as lactic acid bacteria produce hydrogen peroxide metabolically and can be self-inhibited by this system. On the other hand, Gram-negative bacteria such as pseudomonads need an exogenous supply of H_2O_2 to complete the system. Marshall (1978) suggested that the sensitive site in Gram-negative bacteria to the LP system is in the inner cell membrane which led to the inhibition of protein, DNA and RNA synthesis. Reiter & Marshall (1979) found that Cheddar cheese made from milk with glucose, glucose oxidase and SCN, and inoculated with P. fluorescens AR11 (10^5 CFU/ml) had no off-flavour, while the control cheese developed rancid flavour.

It may be feasible to suppress psychrotrophs by the LP

system because it is bactericidal for several Gram-negative bacteria at low temperatures. However, it would be impractical to add glucose and glucose oxidase to milk as a source of H_2O_2 on a commercial scale (Reiter & Marshall, 1979). Bjorck & Rosen (1976) used immobilised β-galactoside (lactase) to generate glucose and immobilised glucose oxidase to generate H_2O_2. The mixed enzyme-associated glass beads were packed in a column. The continuous flow of milk is called a cold sterilisation unit (Figure 1.5.1).

1.5.2.5 Addition of preservatives

Some food preservatives have been studied as growth inhibitors of psychrotrophic bacteria in milk and its products. For example potassium sorbate has been shown to be an effective inhibitor of P. fragi (Moustafa & Collins, 1969); sorbates and benzoates which are preservatives of acidic foods have been found to inhibit a large number of spoilage organisms of cottage cheese (Bonner & Harman, 1957; Saver, 1977). The shelf life of pasteurised milk was increased and the growth rate of psychrotrophs was reduced by adding 0.15% (v/v) potassium sorbate (Mistry & Kosikowksi, 1982).

The present trend towards additive free foods means that in future consumers will be less likely to accept food with additives and preservatives. Therefore, addition of such chemicals may reduce the consumption of milk and its products. Attempts have been made to utilise naturally occurring compounds as antimicrobials. Carbon dioxide has been used to inhibit the growth of psychrotrophs and Pseudomonas spp (Shipe et al., 1982; King & Mabitt[b], 1982).

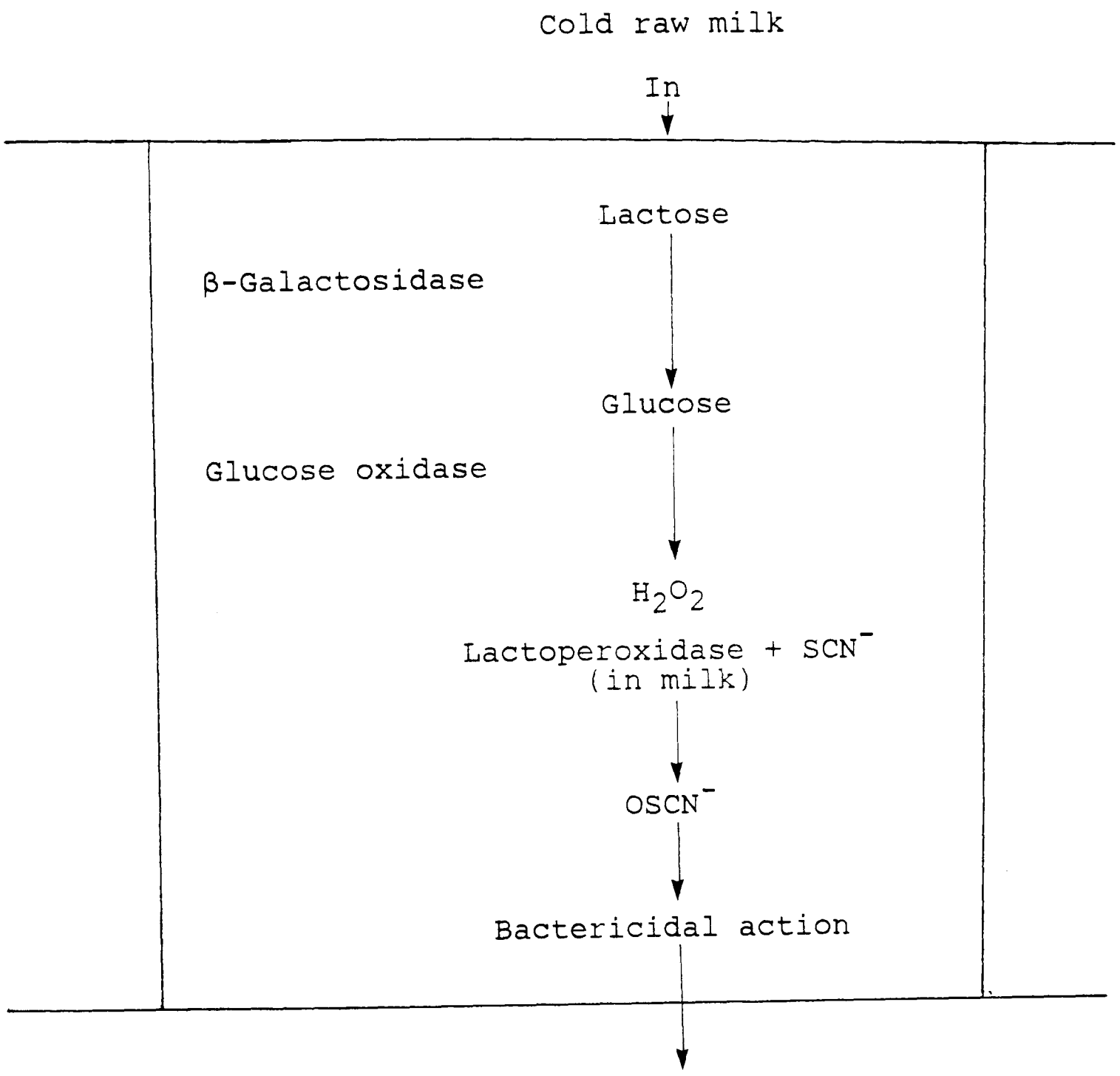


Figure 1.5.1 Flow diagram of "cold sterilisation" unit using the lactoperoxidase system, taken from Law & Mabbitt (1983).

Using carbon dioxide has many advantages; it is cheap, easily removed by warming under vacuum, safe and does not alter the flavour of the product (Mabitt[b], 1982). Law & Mabitt (1983) reported that carbon dioxide could be used to extend the storage time by almost 2 d at 7°C and 3 d at 4°C for the poor quality milk. Another example of the naturally occurring compounds is nitrogen; it has been reported that flushing raw milk with nitrogen leads to a longer lag phase and slower growth rate compared to the control (Murray et al., 1983).

In conclusion it is apparent that none of the previously mentioned methods for controlling the growth of psychrotrophs are widely used. Refrigeration, using carbon dioxide and nitrogen are probably the most acceptable methods because there are fewer disadvantages than with other methods. The former is the most widely used method and the latter may be the alternative for the future.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals and media

All the chemicals used throughout this work, unless otherwise stated were obtained from Sigma (London) Chemical Company Ltd. or British Drug Houses (Macfarlane Robson, Glasgow). Bacteriological media were obtained from Oxoid Ltd. (Basingstoke) and BBL (Becton Dickinson & Co., Cockeysville, MD 21030). Gels for liquid chromatography columns were supplied by Pharmacia Fine Chemicals Ltd. Glass chromatography columns were obtained from Pharmacia and LKB Instruments Ltd.

2.2 Isolation of protease producing psychrotrophic bacteria from raw milk

Samples of bulk milk from various farms in the East of Scotland were obtained from the advisory laboratory of the Edinburgh School of Agriculture. The milk was carefully collected in sterile bottles and kept at 4°C in insulated containers until tested. Standard methods agar (SMA) containing 10% (v/v) reconstituted skim milk (RSM) (American Public Health Association, 1972), sterilised separately and standard methods caseinate agar (SMCA) (Marteley et al., 1970) were used to obtain both psychrotrophic bacterial count (PBC) and proteolytic psychrotrophic count (PPC). Clear zones and white precipitates were seen around the PPC when SMA + RSM and SMCA were used. Samples of milk (1 ml) were removed aseptically and serially diluted into quarter strength Ringer's solution. Aliquots (0.1 ml) of the dilutions were spread using a glass spreader onto SMA + RSM and SMCA plates and incubated at 7°C for 10 days, after which colonies were counted. A similar sample was streaked onto

duplicate nutrient agar (NA) plates and incubated at 30°C for 48 h.

2.3 Maintenance of cultures

Stock cultures of the isolated strains were stored at 4°C on NA slants and subcultured every 2-3 months. The strains were lyophilised following the procedures described by Lapage et al. (1970) using a centrifugal freeze dryer (Edwards model 5PS, Edwards High Vacuum Ltd., Crawley, Sussex).

2.4 Sterilisation

All solutions and growth media except glucose and thermolabile compounds were autoclaved at 15 p.s.i. (121°C) for 15 min. Solutions of thermolabile compounds such as vitamins and glucose were sterilised by filtration (Nuflow membrane filter, 0.22 µm pore size, Oxoid Ltd.).

2.5 Morphological tests

All slides were examined at a magnification of x 1000 under oil immersion using a Vickers microscope.

2.5.1 Gram stain

Gram stains were made, using the method described by Cruickshank (1974), on 24 h old cultures grown in trypticase soy broth (TSB) at 30°C.

2.5.2 Motility

Motility of the cultures was determined using 16 to 24 h cultures grown in TSB, and a hanging drop preparation (Cruickshank, 1974).

2.5.3 Flagella stain

Flagella were examined by transmission electron microscope at 70 KV (Hitachi HU-12A) using the technique

described by Brenner & Horne (1959). The staining procedure was as follows: strains of Pseudomonas fluorescens were grown overnight in peptone-water at 30°C before fixation with 0.4% (v/v) formaldehyde for 1 h. Cells were pelleted by centrifugation at 2 850 g for 30 min and resuspended in 1 M ammonium acetate at pH 7.2. Equal volumes of specimen and 2% (v/v) phosphotungstic acid at pH 7.2 were mixed on the surface of a clean glass slide. A drop of the suspension was transferred by means of a fine platinum loop onto the surface of a formvar coated copper electron microscope grid. After 30 s excess fluid was removed by allowing a piece of filter paper to come into contact with the edge of the specimen drop, and the specimen was dried in a desiccator over anhydrous $CaCl_2$. The specimens were viewed in the transmission electron microscope.

2.6 Biochemical and physiological studies

All the tests in this study were carried out in duplicate using isolated colonies. Uninoculated controls were used in each test where necessary to ensure absence of contamination and provide a negative reference.

Pure cultures of Escherichia coli, Streptococcus lactis and Pseudomonas fluorescens were obtained from the teaching laboratory of the Edinburgh School of Agriculture and were used as reference strains.

2.6.1 Catalase test

One ml of hydrogen peroxide solution (10 vol) was poured over a 24 h old culture of the test organism on NA slopes. The production of gas bubbles from the surface of the solid culture was recorded as a positive result.

2.6.2 Oxidase test

Cultures were grown on nutrient agar plates for 48 h at 30°C. A freshly prepared 1% (w/v) solution of tetra-methyl-p phenylene-diamine dehydrochloride was poured onto the plate engulfing the surface and then decanted. Development of a purple colour was recorded as a positive result.

2.6.3 Anaerobic growth

Two methods were used to assay anaerobic growth of the isolated strains.

2.6.3.1 Thioglycollate medium

The medium described by Brewer (1940) was used. It contained (g l^{-1}): yeast extract, water soluble, 5; casein hydrolysate, pancreatic digest, 15; glucose, 5.5; L-cystine, 0.5; Oxoid agar (No 1), 0.75; NaCl, 2.5; sodium thioglycollate, 0.5 and resazurin sodium solution (0.1%, v/v), freshly prepared, 1.0 ml. The pH of the medium was adjusted to pH 7.3. Duplicate test tubes were inoculated with the strain to be tested and incubated at 30°C for 48 h.

2.6.3.2 GasPak anaerobic technique

A single line streak of each isolate was made on NA medium, the plates were placed in an anaerobic jar (Gallenkamp & Co. Ltd.) and a GasPak envelope was placed (as described by the manufacturer) in the anaerobic jar. The jar was incubated at 30°C for 48 h.

2.6.4 Oxidation fermentation (O/F) test

The oxidation fermentation test was performed as described by Hugh and Leifson (1953) using medium contain-

ing (g l^{-1}): peptone, 2; NaCl, 5; K_2HPO_4, 3; agar, 3 and 1% (v/v) aqueous bromothymol blue, 3.0 ml. The pH was adjusted to pH 7.1 before adding bromothymol blue and then autoclaved. Glucose was sterilised separately and added to give a final concentration of 1% (w/v). The medium was placed in test tubes (14 x 1.5 cm) to a depth of approximately 4 cm. Duplicate test tubes of media for each culture were stab inoculated with a 24 h old culture grown at 30°C. A comparison of aerobic and anaerobic growth was made by covering one of the tubes with 4-5 mm of sterile paraffin followed by incubation at 30°C for 48 h. Production of acid (colour change to yellow) and gas were noted.

2.6.5 Acid production from glucose

The ability of the isolated strains to ferment glucose was tested using peptone water medium containing (g l^{-1}): peptone, 10; NaCl, 10 and 0.2% (v/v) bromothymol blue aqueous solution, 15 ml. The pH was adjusted to pH 7.2-7.3. Sterile glucose solution was added aseptically to peptone water after autoclaving to give a final concentration of 1% (v/v). The medium was placed (5 ml) in test tubes (12 x 1 cm) containing inverted Durham's tubes. Duplicate tubes were inoculated with a drop of liquid culture and incubated at 37°C for 2-4 days. Any colour change and production of gas were recorded.

2.6.6 Production of fluorescent pigment

The production of fluorescent pigment was tested on medium B developed by King et al. (1954). Pigment formation on this medium was checked after 48 h at 30°C using a UV cabinet (366 nm, Ultra Violet Products Inc., Sancabiel,

California, USA).

2.7 Analytical profile index (API)

A standardised micromethod for the identification of non-enteric Gram-negative rods, the API20NE system (API SA, La balme les grottes, 38390 Montalleu Vercieu, France) was performed according to the manufacturer's instructions. This system combines 8 conventional tests and 12 assimilation tests. The conventional tests were inoculated with a bacterial suspension in saline which reconstitutes the media. During the incubation period bacterial metabolism produced colour changes that were either spontaneous or developed upon addition of a reagent.

For assimilation tests 200 µl (4 drops) of the remaining saline solution was added to an ampoule of API20ne medium (Figure 2.1). The tubes and cupules were filled with this medium and incubated at 30°C for 18-24 h.

The reactions were read according to the interpretation table (Table 2.1) and the identification was obtained by the analytical profile index.

2.8 Growth media

2.8.1 Basal medium

Growth in liquid culture was carried out using a modification of the defined minimal medium described by Morihara (1964) and McKellar (1982) containing (g l^{-1}): KH_2PO_4, 0.68; NaCl, 0.10; $MgSO_4.7H_2O$, 0.5; Tris-HCl, 32.5; sodium pyruvate, 2.25; Na_2SO_4, 7.1; $CaCl_2.2H_2O$, 0.294 (separately sterilised); NH_4Cl, 0.80; pH 7.5.

A trace elements solution (3 ml l^{-1}) was added to the basal salts medium (Whooley et al., 1983) and contained (g

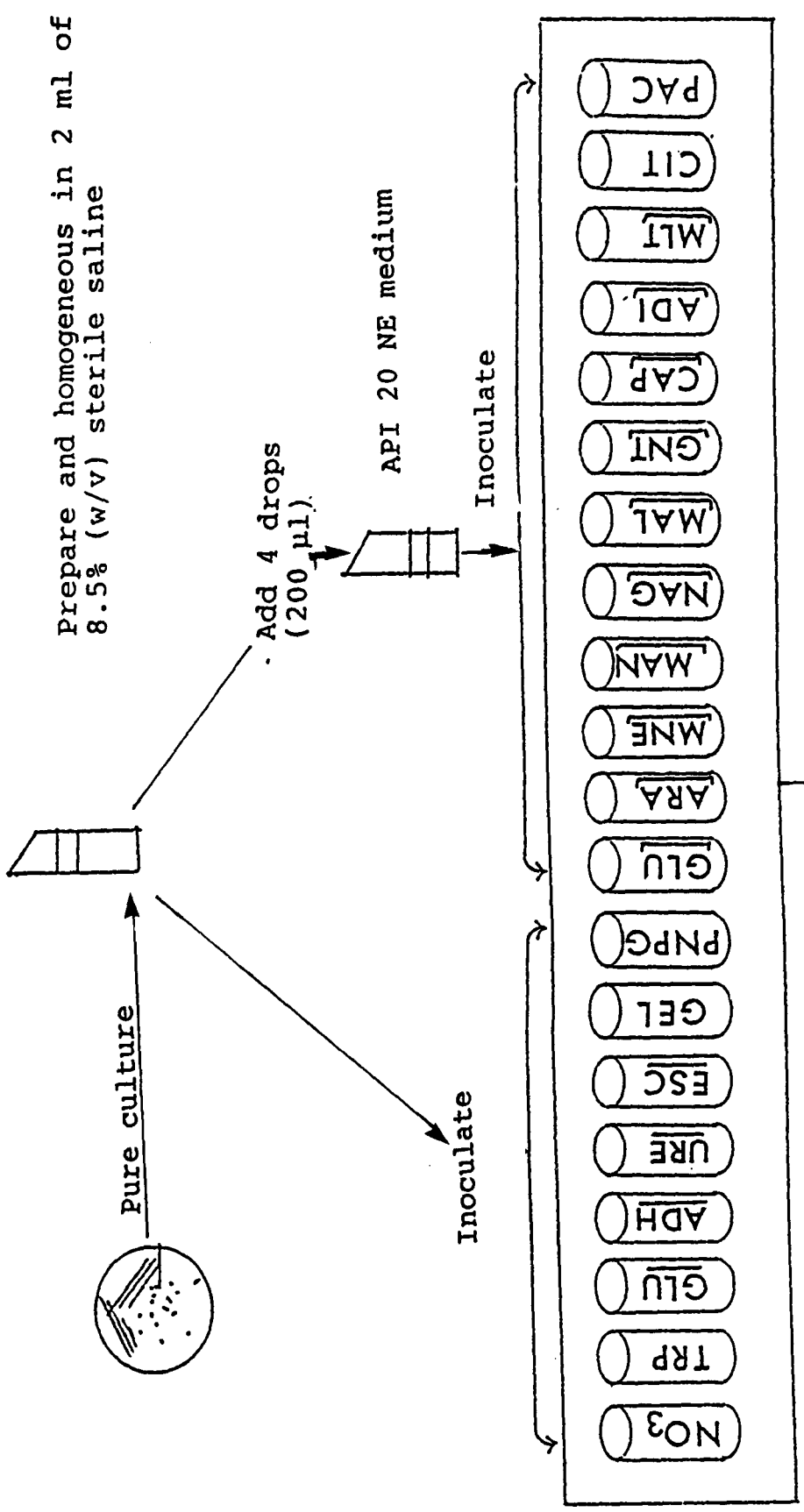


Figure 2.1. Flow diagram of using the API20NE technique for the identification of non-enteric Gram-negative rods

Table 2.1 Interpretation table

Tests	Substrates	Reactions/ enzymes	Results	
			Negative	Positive
		Reduction of nitrates to nitrites	NIT1 + NIT2 15 min	
			Colour-less	Pink-red
NO_3	Potassium nitrate	Reduction of nitrates to nitrogen	Zn/ 5 min	
			Pink	Colour-less
TRP	Tryptophan	Indole production	TRP/ 3-5 min	
			Yellow	Red
Glu	Glucose	Acidification	Blue or green	Yellow
ADH	Arginine	Arginine dihydrolase	Yellow	Orange/ pink/red
URE	Urea	Urease	Yellow	Orange/ pink/red
ESC	Aesculin	Hydrolysis (β-glucosidase)	Yellow	Grey/ brown/ black
GEL	Gelatin	Hydrolysis (protease)	No pigment diffusion	Diffusion of black pigment
NPPG	p-Nitrophenyl-BD-galatopyranoside	β-glactosidase	Colour-less	Yellow
Glu	Glucose	Assimilation	Trans-parent	Opaque
ARA	Arabinose	Assimilation	Trans-parent	Opaque
MNE	Mannose	Assimilation	Trans-parent	Opaque
MAN	Mannitol	Assimilation	Trans-parent	Opaque
NAG	N-acetyl-glucosamine	Assimilation	Trans-parent	Opaque

Table 2.1 (cont'd)

Test	Substrates	Reactions/ Enzymes	Results	
			Negative	Positive
MAL	Maltose	Assimilation	Trans-parent	Opaque
GNT	Gluconate	Assimilation	Trans-parent	Opaque
CAP	Caprate	Assimilation	Trans-parent	Opaque
ADI	Adipate	Assimilation	Trans-parent	Opaque
MLT	Adipate	Assimilation	Trans-parent	Opaque
CIT	Citrate	Assimilation	Trans-parent	Opaque
PAC	Phenyl-acetate	Assimilation	Trans-parent	Opaque

l^{-1}: $FeNH_4(SO_4)_2.12H_2O$, 0.0142; H_3BO_3, 0.232; $CoCl_2.6H_2SO_4$; $CuSO_4.5H_2O$, 0.008; $MnSO_4.4H_2O$, 0.008; $(NH_4)_6MO_7O_{24}.4H_2O$, 0.22; $ZnSO_4.7H_2O$, 0.174. When required the basic medium was supplemented with the following:

1. Individual amino acids at a final concentration of 40 µg ml^{-1}.
2. A mixture of the 20 amino acids each at a final concentration of 20 µg ml^{-1}.
3. A vitamin mixture was used at the following final concentrations of individual vitamins (mg ml^{-1}): bioin (10); thiamin (HCl) (10); nicotinic acid (500); folic acid (500) and panthothenic acid (500).
4. Different carbon sources such as succinate, malate, pyruvate, glucose, aspartate and glutamate were used at a final concentration of 40 mM.

2.8.2 Complex medium

Trypticase soy broth (BBL) or TSB supplemented with 1% (w/v) skim milk was used as a complex medium.

2.8.3 Growth in minimal medium

For each growth experiment on minimal medium strains grown on NA slopes were inoculated into trypticase soy broth (50 ml in 250 ml conical flasks) and grown overnight with shaking in a Gallenkamp orbital cooled shaker at 120 rev/min. Minimal medium (50 ml) was inoculated with 0.1 (v/v) of the culture grown overnight. Once the end of the logarithmic phase or the beginning of the stationary phase had been reached a second subculture was then carried out into the test medium at 10% v/v.

2.8.4 Growth in complex medium

Strains grown on NA were inoculated into 50 ml TSB and grown at 20°C with shaking and the same steps were performed as described above.

2.9 Measurement of bacterial growth

Growth of bacterial cultures was monitored by the increase in absorbance at 660 nm (A660) in a 1 cm path length cuvette using a single beam spectrophotometer (Shimadzu UV 120.02).

2.9.1 Dry weight

Pseudomonas fluorescens R8 was inoculated into a series of 2 l conical flasks containing either basal medium supplemented with glutamate or typticase soy broth (400 ml). At various intervals the A660 of the culture was determined and the cells harvested by centrifugation at 10 000 for 15 min at 4°C. The cells were resuspended in 10 ml of the growth medium using a hand homogeniser. Duplicate 5 ml volumes of the concentrated cell suspension were pipetted into porcelain containers which had been dried at 80°C for 2 d in a hot air oven and cooled in a desiccator. The porcelain containers which had the concentrated cell suspensions were dried in a hot air oven for a further 2 d and cooled in a desiccator. The porcelain containers were dried to constant weight. The dry weight of the sample was calculated taking into account the concentration factor and corrected for the dry weight of the blank sample containing 5 ml of growth medium.

2.10 Preparation of protease extracts

Strains of P. fluorescens grown initially on trypti-

case soy broth were inoculated at 1% (v/v) into flasks containing 400 ml TSB supplemented with 1% (v/v) skim milk. The flasks were incubated at 20°C with shaking for 72 h.

2.11 Harvesting and preparation of supernatants

The protease was harvested from the culture medium by centrifugation at 17 000 g in a Sorval B centrifuge for 30 min at 4°C using 300 ml acid washed plastic bottles.

2.12 Concentration of the culture supernatant

2.12.1 Ultrafiltration

Concentration of samples by ultrafiltration was performed using the Millipore Pellican cassette system (model no XX 4200080) and 10 000 daltons cut-off membrane filter. The system was pressurised with nitrogen (20 psi).

2.12.2 Dialysis

Concentration of the supernatant was performed by using dialysis tubing (size 8). Tubing was boiled in 10 mM EDTA disodium salt and stored at 4°C. Before use, the tubing was washed with distilled water inside and outside. Dialysis of the enzyme solution or the supernatant was performed overnight against 20% (w/v) polyetheleneglycol (PEG, 6,000 MW), or by applying solid PEG around the tubing (Scopes, 1982). After concentration against PEG the samples were redialysed against an appropriate buffer to equilibrate salts.

2.12.3 Microcentration

Small volumes of the culture supernatant when R8 was grown in basal medium in the absence of $CaCl_2$ were concentrated using a microcentration method. Culture supernatant was concentrated twice firstly using PEG (Section 2.12.2)

and then using Amicon microcentrator cups (Amicon Ltd., Upper Mill, Stonehouse, Glos, UK). Samples were concentrated from 2.0 ml down to 140 µl.

2.13 Ammonium sulphate precipitation

Ammonium sulphate fractionation of concentrated preparation was performed according to Green & Hughes (1955) using solid ammonium sulphate (specially low in heavy metals for enzyme work). Ammonium sulphate was gradually added with stirring at 4°C over 20 min. Stirring was continued for a total of 2 h and the precipitate was separated by centrifugation at 30 000 g (Sorval RC5B, 8 x 50 ml rotor) for 30 min at 4°C. The precipitate was redissolved in 10 ml of 50 mM Tris HCl, pH 8.0.

2.14 Protein determination

Protein concentrations were assayed by the Bio-Rad dye-binding method using either the macro- or the microassay procedure, as appropriate (Bradford, 1976). Bovine serum albumin supplied by Bio-Rad was used as the standard protein. Typical calibration curves are given in Figures 2.2 and 2.3.

2.15 Liquid chromatography

All procedures were carried out at 4°C. Flow was provided by peristaltic pumps (Pharmacia P1).

2.15.1 Gel filtration

Two gel types were used as gel filtration media: Sephadex G-75 and Sephacryl S-300 superfine in columns of bed dimensions 1.6 x 100 cm. The columns were equilibrated in gel filtration buffer (50 mM Tris HCl, pH 8.0 containing 200 mM NaCl). Samples were loaded in a volume not exceed-

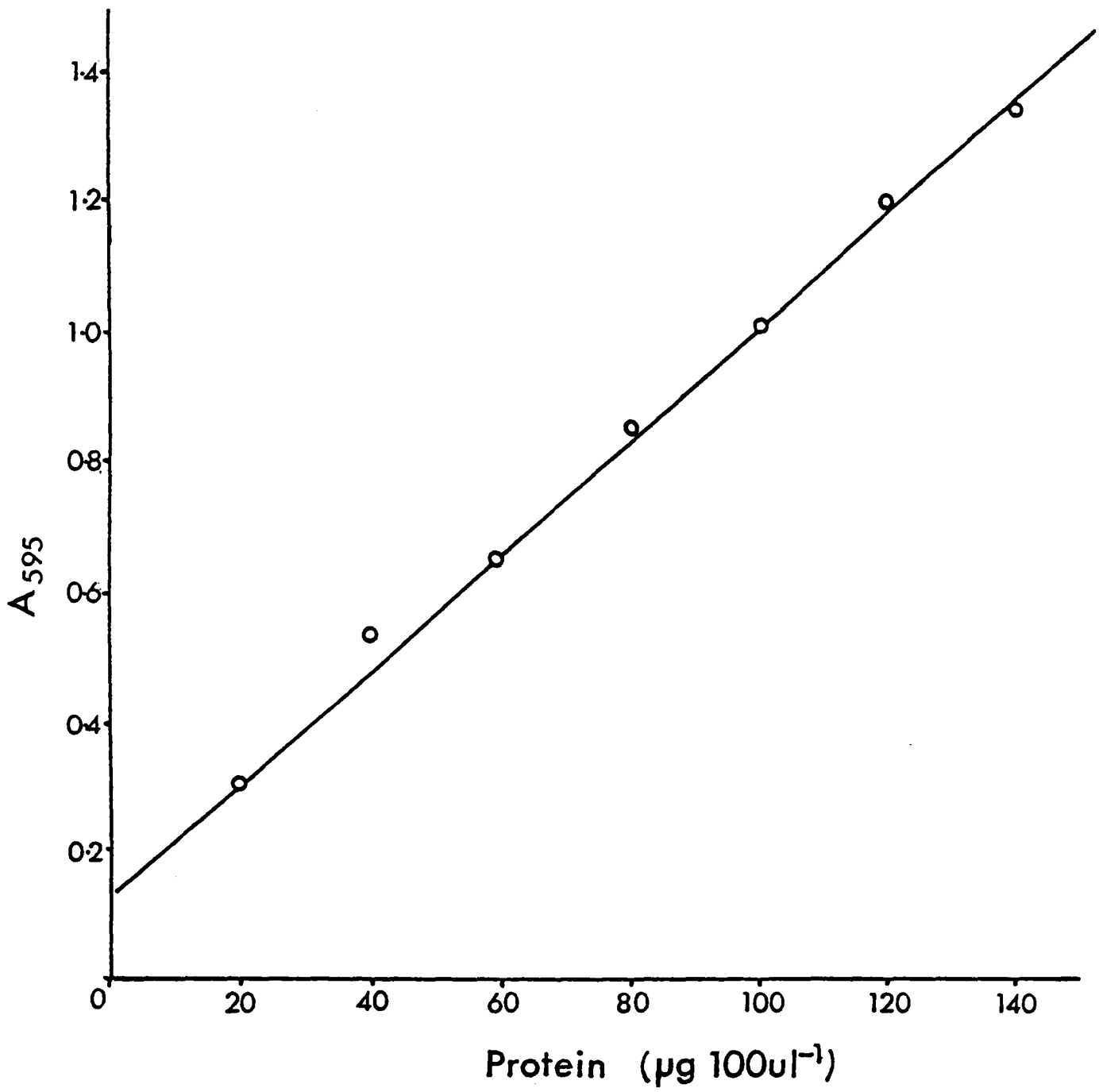


Figure 2.2 Bio-Rad calibration curve (macroassay)

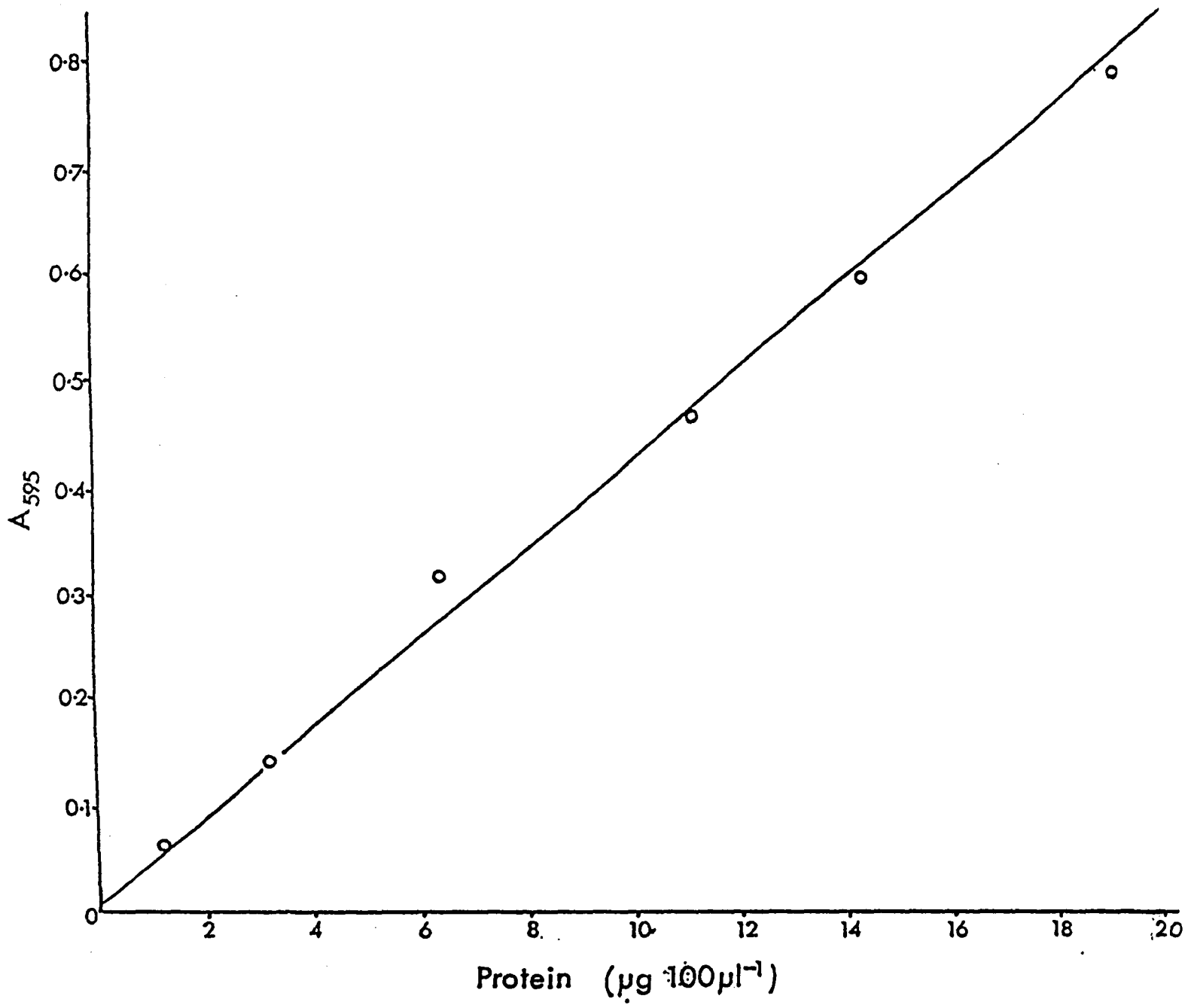


Figure 2.3 Bio-Rad calibration curve (microassay)

ing the recommended limit of 5% column volume and proteins were eluted isocratically with gel filtration buffer. The eluate was collected in acid-washed test tubes using an Ultrorac Fraction Collector (LKB) and samples were assayed for protease activity as in Section 2.18 and for protein by absorbance at 280 nm.

The molecular weight calibration curve for proteins eluted from the gel filtration columns was prepared using a series of molecular weight standard proteins: β-amylase (200K daltons), bovine serum albumin (BSA, 66K daltons), ovalbumin (45K daltons) and cytochrome c (12.5K daltons). All standards were prepared as 5 mg ml^{-1} solutions and loaded in 1 ml aliquots. Blue dextran (Mr = 2 x 16^6 daltons) was used to determine the void volume (Vo) of the Sephacyl S-300 column. The calibration curve was plotted as Kav (partition coefficient) against $\log_{10}$ Mr, where

$$Kav = \frac{Ve - Vo}{Vt - Vo}$$

Ve = elution volume of protein and Vt = total column volume.

2.15.2 Ion exchange chromatography

The anion exchange DEAE Sepharose CL6B was packed into a column of bed dimensions 2.6 x 10 cm. The gel was equilibrated with the ion exchange buffer (50 mM Tris HCl, pH 8.0). After loading the sample the column was washed with three column volumes to remove unbound protein. Bound protein was eluted with an increasing linear gradient of NaCl in the same buffer (400 ml). Fractions were collected and assayed as described previously in Section 2.15.1.

2.15.3 Hydrophobic interaction chromatography

Phenyl Sepharose was used in a column of bed dimensions 2.6 x 10 cm. The gel was equilibrated with 50 mM Tris-HCl pH 8.0 containing 4 M NaCl. Samples were prepared for chromatography by dialysis against the column buffer containing 4.0 M NaCl. Bound protein was eluted with a decreasing linear gradient of NaCl (4.0 M-2.0 M and 2.0 M-0.0 M) in 50 mM Tris-HCl pH 8.0.

2.16 Polyacrylamide gel electrophoresis

2.16.1 Electrophoresis conditions

Polyacrylamide gel electrophoresis (PAGE) in the absence and presence of sodium dodecyl sulphate (SDS-PAGE) was carried out using the method described by Laemmli (1970). Stock acrylamide solution was prepared from acrylamide (29.2% w/v) and NN'methylenebisacrylamide (0.8% w/v) in aqueous solution and stored at 4°C in the dark. Linear gradient resolving gels (7.5-15%) were prepared by using a gradient mixer. Gels were cast as 1 mm slabs with a 20 x 14 cm resolving gel and a 2.5 x 14 cm stacking gel. Purified and partially purified preparations were electrophoresed on 12.5% or gradient gels (7.5-15%) as appropriate.

Samples for electrophoresis were prepared in sample buffer (10 mM Tris HCl, pH 6.8 containing: glycerol, 10% (v/v); SDS ±, 1% (w/v); ± dithiothreitol, 5% (w/v) and bromophenol blue, 0.005% (w/v) as tracking dye).

Electrode buffer contained (g l^{-1}): Tris-base, 3; glycine, 14.4; ± SDS, 1.0; pH 8.3. Electrophoresis was carried out at 10 mA (LKB model 2197 power supply).

Samples were loaded in 5-100 µl aliquots and the gel run at 10 mA constant current for 1 h followed by 20 mA constant current until the tracking dye migrated to 1 cm above the base of the gel (4-6 h). Gels were stained for protein or enzyme activity following electrophoresis. All electrophoretic separations were carried out at room temperature.

2.16.2 Kenacid blue stain for proteins

Kenacid brilliant blue R (1.25 g) was dissolved in methanol (227 ml), glacial acetic acid (46 ml) and water (227 ml). The dye solution was filtered through Whatman No 1 filter paper to remove any insoluble material before use. Polyacrylamide gels were placed in the staining solution and incubated with shaking for 2 h or statically overnight. Excess stain was removed and the gels were destained in a destaining solution containing methanol (50 ml), glacial acetic acid (75 ml), water (875 ml) and stored in 5% (v/v) acetic acid.

2.16.3 Silver stain for protein

Ultrasensitive staining for proteins on polyacrylamide gels was performed using the Bio-Rad silver stain method explained in Table 2.2.

2.17 Zymogram stain for protease activity

Detection of protease activity after PAGE was carried out using two methods. The first method was described by Himelbloom (1986, personal communication); native gels immediately after electrophoresis were placed on the protease detection medium containing: 1% (w/v) sodium caseinate; 0.5% (v/v) tryptone; 0.25% (w/v) yeast extract; 20 mM $CaCl_2$ and 1.5% (w/v) agar in 15 mM Tris HCl

Table 2.2 Method for Bio-Rad silver staining of protein

A. Solutions. The following solutions were prepared freshly for each gel using "Analar" grade chemicals.

a) Fixative A: 40% (v/v) methanol, 10% (v/v) acetic acid in distilled water.

b) Fixative B: 10% (v/v) ethanol, 5% (v/v) acetic acid in distilled water.

c) Oxidiser: potassium dichromate, 0.2 g in 200 ml distilled water containing 0.04 ml concentrated nitric acid.

d) Silver reagent: silver nitrate, 0.4 g in 200 ml distilled water.

e) Developer: sodium carbonate, 18 g in 600 ml distilled water containing 0.30 formaldehyde.

f) Stop: 0.5% (v/v) acetic acid in distilled water.

B. Protocol. Reagents were added in the order given below.

Reagent		Volume (ml)	Incubation time
1.	Fixative A	400	30 min/overnight
2.	Fixative B	400	15 min
3.	Fixative B	400	15 min
4.	Oxidiser	200	5 min
5.	Distilled water	400	5 min
6.	Distilled water	400	5 min
7.	Silver reagent	200	20 min
8.	Distilled water	400	1 min
9.	Developer	200	30 s
10.	Developer	200	30 s
11.	Developer	200	Until developed
12.	Stop solution	400	For storage

pH 7.0. The gels were incubated at 37°C for 12-18 h. Clear zones showed the areas of protein hydrolysis. The second method was described by Cliffe & Law (1985). The gel was immediately immersed in Hammarsten casein solutions 2.5% (w/v) at pH 6.0 and 7.0 for 30 and 45 min then rapidly washed twice with water before being stained in Kenacid brilliant blue as described in Section 2.16.2.

After staining hydrolysis of casein could be seen as clear zones, thus indicating the presence of proteolytic activity.

2.18 Protease assay

Enzyme activity was measured by the hide powder azure (HPA) and azocasein methods.

2.18.1 The hide powder azure method

The method was that of Cliffe & Law (1982) using an assay mixture consisting of HPA, 18 mg; Tris HCl (20 mM) pH 8.3 (1.2 ml) and 0.3 ml of the enzyme solution.

2.18.2 The azocasein assay

The second assay was a modification of the method described by Jensen et al. (1980c). The assay mixture contained 1.0 ml of azocasein reagent (0.5%, w/v solution in distilled water adjusted to pH 7.5 and filtered through Whatman No 1 filter paper), 1.0 ml of 0.02 M Tris HCl pH 7.5 containing 0.002 M $CaCl_2$ and 0-10 µl of the enzyme solution. The reaction mixture was stopped after 15 min of incubation at 37°C by the addition of 2.0 ml of 10% (v/v) TCA. The precipitate was removed by centrifugation (Eppendorf centrifuge 7 500 g for 10 min). The absorbance of the supernatant was measured at 370 nm. One unit of activity

was defined as the amount of enzyme needed to produce an increase in absorbance at 370 nm of 1.0 in 1 min under standard assay conditions.

2.19 Immunoelectrophoresis

2.19.1 Preparation of antiserum against the purified protease

Antibodies against the purified protease (peak A) were raised in 10 mice and 2 rabbits. Before immunisation a sample of blood was taken from each animal (0.2 ml) as a pre-immune serum control. Mice and rabbits were immunised with a primary dose of purified protease (50 µg per mouse and 100 µg per rabbit) in equal parts complete Freund's adjuvant and 2% (v/v) Tween 80 (total volume 400 µl) and administered in the abdomen. On weeks 6, 10 and 14 after the primary dose, mice and rabbits were given a booster dose of antigen (75 µg of purified protease to each animal) administered as previously but with incomplete Freund's adjuvant. On weeks 7, 11 and 19 samples of blood (0.25 ml from each mouse and 15 ml from each rabbit) were taken and the serum separated from cellular material by centrifugation (5 000 g, 10 min, MSE Centaur bench centrifuge). The antiserum was not purified further and was stored in aliquots of 25 µl at -20°C.

2.19.2 Equipment, reagents and general procedures

All immunoelectrophoresis methods were based on those of Axelsen et al. (1973). Agarose immunoelectrophoresis was carried out using an multiphor flat-bed electrophoresis tank (LKB model 2197 power supply). Whatman No 1 filter paper was used for the wicks (20 x 14 cm), which were pre-

soaked in tank buffer. The latter was made up as a 40% (v/v) solution of stock buffer which contained (g l^{-1}): Tris-base, 27.26; glycine, 131.5; Triton X-100 (25 ml). Gels for electrophoresis were prepared from 3.3 ml 1% (w/v) agarose (Mercia Brocades) in tank buffer. Glass slides (5 x 5 cm) were cleaned, washed with ethanol, dried and coated with a thin layer of 1% (w/v) aqueous agarose. This was important to ensure good adhesion between the gel and the plate during the washing and staining procedure.

Gel casting was carried out on a horizontal table, to ensure a uniform gel. Agarose (3.3 ml) in tank buffer was melted and boiled at 100°C. The tube was transferred to a water bath at 55-58°C. After a minimum of 2 minutes at this temperature the molten agarose was poured onto the coated glass plate. Air bubbles were removed with a hot needle and the agarose allowed to set.

2.19.3 Rocket immunoelectrophoresis (RIE)

Agarose (3.3 ml) in tank buffer was melted and placed at 55-58°C for 2 min. Antiserum (25 µl) was added and the contents of the tube mixed for 2 min. The gels were poured as described in Section 2.19.2 above. Wells (7) were cut at approximately 0.5 cm intervals across one edge of the gel 1 cm from the bottom.

The gels were then placed in an immunoelectrophoresis tank and wells filled with 6 µl samples either in H_2O or in H_2O containing 10 mM EDTA with a current of 7 mA (50 volts) passing through. This was necessary to ensure that samples did not diffuse radially into the agarose during loading but moved out of the well towards the anode. The current

was then increased to 14 mA (100 volts) and maintained for 16-18 h at 10-15°C. The gels were washed, stained and destained as described in Section 2.19.4 and the peak height of rockets was measured.

2.19.4 Washing, staining and destaining

Gels were washed for 20 min in 0.1 M NaCl to remove unprecipitated proteins. Gels were covered with layers of Whatman No 1 filter paper. A glass plate and a weight (250 g/gel) were placed on top of the filter paper. Air bubbles between gel and paper were removed by gentle pressing before addition of weights. The paper was removed after 10 min by flooding the gels with H_2O and gently peeling away the filter paper, leaving the gel on the plate. This process was repeated twice with a final wash for 15 minutes in H_2O to remove NaCl. The gel was dried to a fine film on the glass plate in a stream of warm air.

Dried gels were stained for 10 min, in 0.5% (w/v) Coomassie brilliant blue R250 or Kenacid blue R in 45% (v/v) ethanol, 10% (v/v) acetic acid in H_2O. Excess stain was removed by gentle blotting with filter paper and the gels destained in 45% (v/v) ethanol, 10% (v/v) acetic acid in H_2O. Gels were then redried on a hot-plate and stored.

2.19.5 Tandem crossed immunoelectrophoresis (TCIE)

This method allows the immunochemical comparison of two samples by the method of crossed immunoelectrophoresis. All equipment and reagents were as in Section 2.19.2. Agarose gels were cast as described in Section 2.19.2 and two wells cut into the bottom left hand corner, 0.5 cm

apart (from the centre of each well) and 1.0 cm from the edge of the gel. The two wells were loaded with the two samples and these were allowed to diffuse completely into the agarose. Prior to running, the wells were sealed with a drop of molten agarose. Gels were run in the first dimension using a current of 20-30 mA (150-200 volts) for 45 min in the negative to positive direction. After running in the first dimension, a 3.5 x 5 cm area of agarose was removed from the gel leaving a 1.5 cm x 5.0 cm strip up the left side including the wells in the bottom left hand corner. An agrose gel (2.3 ml) containing antiserum was cast onto the remainder of the gel and electrophoresed under the same conditions as for RIE (Section 2.19.3 and 2.19.4).

2.20 High performance liquid chromatography (HPLC)

An LKB HPLC system was used, consisting of two HPLC pumps, controller, gradient mixer and Uvicord fixed wavelength (280 nm) UV detector.

2.20.1 Gel permeation liquid chromatography (GP-HPLC)

Gel permeation HPLC was carried out using a TSK G2000 SW 30 cm gel filtration column with an Ultrapac TSK GSWP guard column (LKB, Broma, Sweden). Phosphate buffer (0.1 M Na_2HPO_4/NaH_2PO_4 pH6.8) was filtered and degassed using 0.45 µM Durapore (Millipore) filters. Protein samples (1 mg ml^{-1}) in phosphate buffer were filtered through HV4 filter units (Millipore) prior to injection in 100 µl aliquots through a Rheodyne 7125 injection valve. The flow rate was 0.8 ml min^{-1}. Ovalbumin (45K daltons), carbonic anhydrase (29K daltons) and bovine serum albumin (67K

daltons) were used as standards for the calibration curve. All standards were prepared as 2 mg ml^{-1} protein.

2.20.2 Reversed-phase high-performance liquid chromatography (RP-HPLC)

RP-HPLC was carried out using an Apex C8 120°A column and C8 guard column (Jones Chromatography, Llanbradach, UK). Solvent A was 0.1% (v/v) trifluoroacetic acid (TFA) (pH 2.1) and solvent B was 0.08% TFA (v/v) in 50% (v/v) acetonitrile (pH 2.3). All solvents were filtered as described above. Samples for RP-HPLC were dissolved in either solvent A or in solvent containing 10% (v/v) acetonitrile (pH 2.1). All samples (40-100µg) were filtered prior to injection. Flow rate was 0.8 ml/min. Samples were eluted using a gradient of increasing acetonitrile at 1%/min.

2.21 Determination of metal content

Calcium, zinc, iron and copper were determined using a Instrumentation Laboratory atomic absorption spectrophotometer 157. Absorbance was measured at 248.3, 422.7, 213.9 and 324.7 nm for iron, calcium, zinc and copper respectively. Enzyme samples were extensively dialysed against deionized water. Standard curves were obtained from a range of suitable standards.

2.22 Amino acid analysis

An enzyme solution (0.75 mg protein in 50 mM Tris HCl pH 8.0) was freeze dried and 0.4 ml 6 N HCl added. The vial was sealed under vacuum and hydrolysed at 105°C overnight. The sample was redried and redissolved in sodium citrate buffer, pH 7.2 containing internal standards and

loaded onto a Beckman 120°C amino acid analyser.

2.23 N-terminal residue

N-terminal residues were determined by a dansylation technique (Allen et al., 1981). Separation of the dansyl amino acids was in a flat-bed electrophoresis system at pH 4.4 using pyridine:acetic acid:water (9:15:976).

2.24 Determination of protease pH optimum

Samples (10 µl of pure protease in 20 mM Tris HCl pH 7.5 were added to assay mixtures containing 1 ml azocasein (0.5%, w/v in water) and 1 ml of the different test buffers (50 mM): acetate (pH 3.6-5.6), Tris maleate (pH 5.6-6.6), Tris HCl (pH 6.6-8.6), glycine-NaOH (pH 8.6-10.4). All the buffer solutions were prepared as described by Dawson et al. (1986). Protease activity was then assayed as previously described in Section 2.18.2.

2.25 Determination of protease temperature optimum

Azocasein substrate solution (1 ml, 0.05% w/v in water) and 1 ml Tris HCl buffer pH 7.5 were added to a series of assay tubes. The tubes were then equilibrated in a thermostatically controlled water bath (Grant type SU5) at various temperatures. After incubation 10 µl of the protease in 20 mM Tris HCl pH 7.5 was added to the assay tubes and the mixtures reincubated for a further 15 min. The protease activity was then assayed as previously described in Section 2.18.2.

2.26 Carbohydrate content

Total neutral sugars in the purified enzyme were quantitatively determined by the phenol H_2SO_4 method of Dubois et al. (1956) using mannose as a standard.

2.27 Heat stability

Solutions of purified protease (200 µl) in 20 mM Tris HCl pH 7.5 were sealed in 12.5 x 0.2 cm capillary tubes, the tubes being filled about two-thirds full. The tubes were then subjected to the desired time/temperature treatment in a thermostatically controlled silicon oil bath (Lauda K4 Electronic), cooled rapidly in an ice/water mixture, and enzyme activity assayed by the azocasein assay. The heating up time, as determined by a copper thermocouple was not included in the total heating time. A water bath (Grant SU5) was used for temperatures below 90°C. Heat treatments used were 55°C for 1 h, equivalent to low temperature inactivation (LTI), 65°C for 30 min and 73°C for 15 sec, both equivalent to milk pasteurisation.

D-value (time necessary at a given temperature to decrease the enzyme activity to 10% of its original activity) of the purified enzyme was determined at 140°C by heating aliquots of the enzyme as prepared above for various time intervals. After heating, samples were assayed for residual protease activity.

2.28 Determination of the effect of metal ions and inhibitors

Most of the metal ions and inhibitors were dissolved in 0.1 M Tris HCl buffer, pH 7.5. TPCK was dissolved in a 25% (v/v) mixture of ethanol in the same buffer. Where necessary, the pH of each of the inhibitor/activator solutions was readjusted to pH 7.5.

To each assay tube, buffer solutions of inhibitors and metal ions were added to a total volume of 1 ml. Portions

(10 µl) of protease were then added to each assay tube and the mixture incubated at 37°C for 30 min. Azocasein substrate solution (1 ml) was then added to each mixture and protease activity was assayed as previously described.

2.29 Substrate specificity

Substrate specificity of purified protease against casein, β-casein, α_{S1}-casein, α_{S2}-casein, cytochrome c, bovine serum albumin and β-lactoglobulin was determined by a modification of Hull's method (1947) using a final concentration of 1% (w/v) of these substrates. β-Casein, α_{S1}- and α_{S2}-caseins were provided by Dr Leadbeater (Department of Microbiology, Edinburgh School of Agriculture). The substrates were extensively dialysed against Tris-HCl buffer, pH 7.5 to remove free amino acids. A standard reaction mixture (1 ml) contained 0.5 ml of 50 mM Tris-HCl buffer, pH 7.5 with 10 mM $CaCl_2$ and 0.5 ml of protease-substrate mixture. The standard reaction mixture was incubated in a 37°C water bath (Grant, type SU5) for 30 min. The reaction was stopped by the addition of 1.0 ml of 12% (w/v) trichloracetic acid (TCA) solution to the reaction mixture. The precipitated proteins were removed by centrifugation (Eppendorf centrifuge, 7 500 g) for 10 min and the amount of aromatic amino acids in the clear supernatant was determined spectrophotometrically at 280 nm. One enzyme unit is the amount of enzyme that releases 1 µmol of tyrosine per min per ml at 37°C. A calibration curve (Figure 2.4) was constructed between µmol tyrosine ml^{-1} and A280. P-toluenesulphonyl-L-arginine methyl ester (TAME) (trypsin substrate), N-acetyl-L-tyrosine ethyl ester

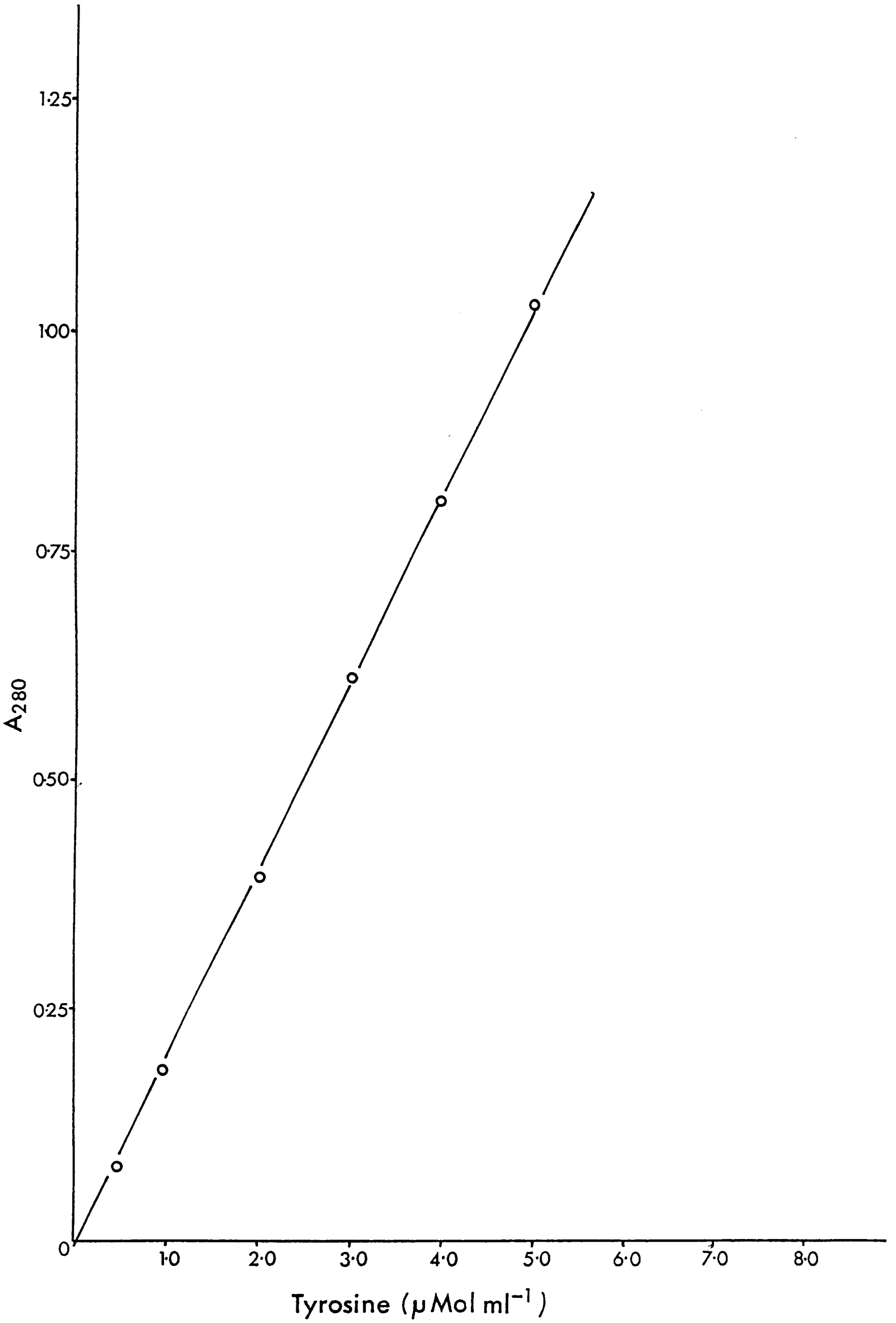


Figure 2.4 Relationship between μmol tyrosine ml^{-1} and absorbance at 280

(ATEE) (chymotrypsin substrate) and N-t-Boc-L-leucine-P-nitrophenyl ester (esterase substrate) were used as synthetic substrates to determine the protease activity using the method described by Walsh & Wilcox (1970).

CHAPTER 3

ISOLATION OF *Pseudomonas fluorescens* R8 AND PRODUCTION OF EXTRACELLULAR PROTEASE

3.1 Isolation and identification of proteolytic psychrotrophic bacteria from raw milk

3.1.1 Isolation and detection of the strains

Samples of bulk milk from six farms in the East of Scotland were used to isolate proteolytic psychrotrophic bacteria (Section 2.2). Standard methods caseinate agar (SMCA) and standard methods agar (SMA) fortified with 10% (w/v) reconstituted skim milk (RSM) were used to detect protease-producing psychrotrophic bacteria. Plate 3.1.1 shows clear zones around most of the colonies grown on SMA + 10% RSM. These clear zones were due to the breakdown of milk proteins. These strains were isolated from a heavily contaminated sample of raw milk as a trial to detect the protease-producing bacteria grown on plates and incubated at 7°C for 10 d. Plate 3.1.2 shows clearly the difference between proteolytic and nonproteolytic colonies grown on SMA + 10% RSM. Instead of the formation of white precipitation zones (mainly para-k-casein) normally clear zones were found around the colonies when SMCA was used (Martely et al., 1970). The explanation put forward by Martely et al. (1970) was that clear zones are due to the extensive degradation of para-caseins. The results obtained in this study indicate that the isolates were strongly active proteolytic organisms.

3.1.2 Total viable count (TVC), psychrotrophic bacteria count (PBC) and proteolytic psychrotrophic count (PPC)

Milk samples were analysed for bacterial counts as described in Section 2.2. Plates containing 30-200

Plate 3.1.1. Proteolytic activity of different colonies on SMA + 10% RSM

Incubation at 7°C for 10 d

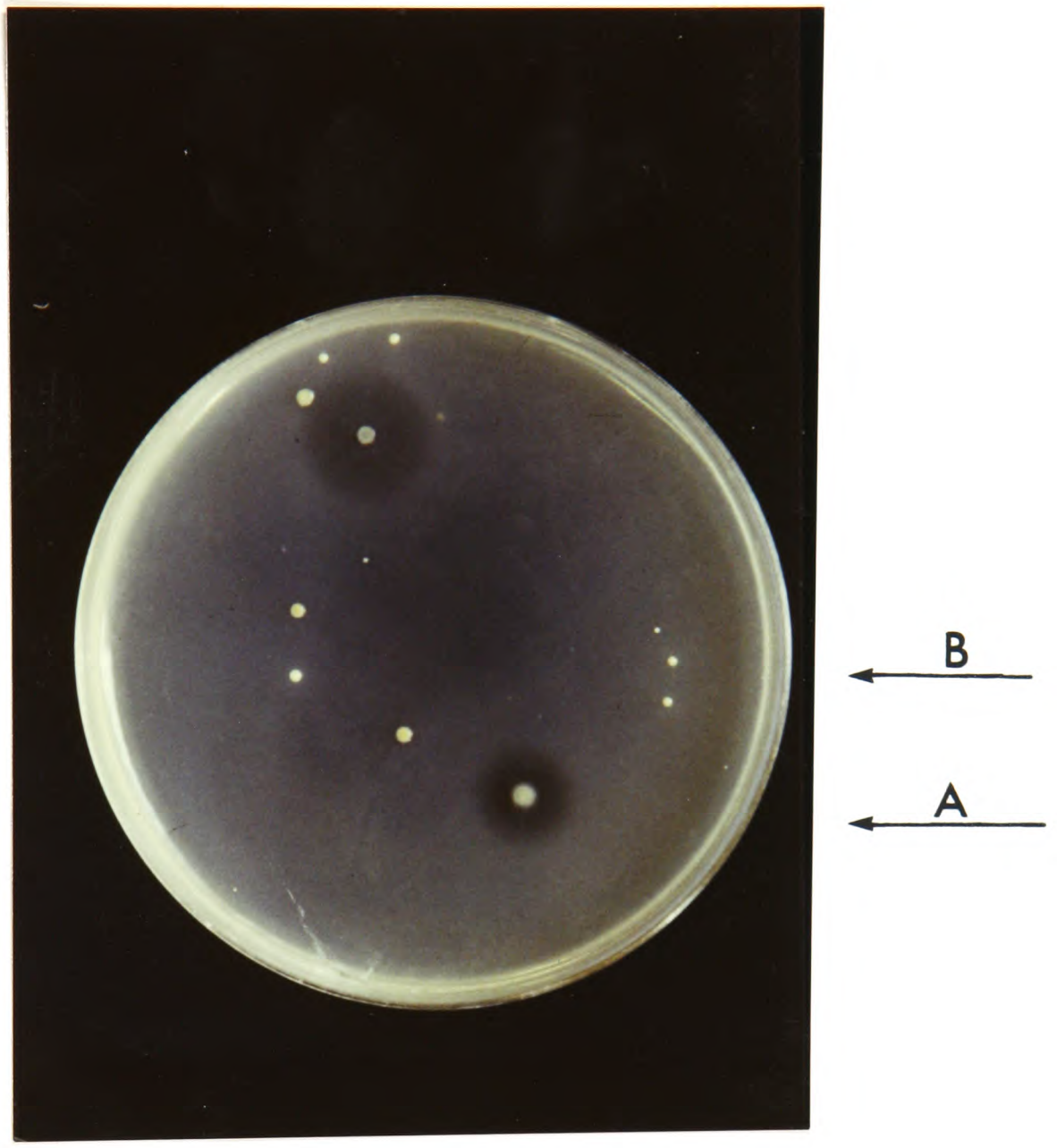


Plate 3.1.2. Differentiation of proteolytic (A) and non-proteolytic (B) colonies on SMA + 10% RSM

Incubation at 7°C for 10 d

colonies were counted and the results were expressed as total viable count (TVC), psychrotrophic bacterial count (PBC) and proteolytic psychrotrophic count (PPC). The data given in Table 3.1.1 show the TVC, PBC and PPC of six raw milk samples. The total viable count obtained in this study was 5.8×10^3-7.2×10^3 ml^{-1}. This figure is lower than obtained by Dwivedi (1976) and Malik & Mathur (1983). Not only TVC was lower in this study but also PBC and PPC were lower than the corresponding counts in the previous studies (Malik & Mathur, 1983). The lower figures obtained in this study compared to those obtained by Dwivedi (1975) and Malik & Mathur (1983) indicate the good bacteriological quality of the milk used. Results given in Table 3.1.2 show that proteolytic psychrotrophic bacteria represented 3.0-26.0% of the total viable count and 21.5-61.0% of the psychrotrophic bacterial count. Previous studies showed that protease-producing psychrotrophic bacteria isolated from raw milk represented between 72-100% of the TVC (White et al., 1978; West et al., 1978) and 72% of the PBC (Richardson & Te Whaiti, 1978).

3.1.3 Preparation of the strains for identification tests

Isolated colonies of bacteria grown on SMA + 10% SM that were surrounded by clear zones were picked and sub-cultured into trypticase soy broth (TSB). After incubation at 30°C for 24 h, a loopful of culture was removed, streaked onto SMA + 10% RSM plates and incubated at 30°C for 48 h to obtained isolated colonies which were rechecked for proteolytic activity. Proteolytic isolates were inoculated into TSB and incubated at 30°C for 24 h. The

Table 3.1.1 Total viable count (TVC), psychrotrophic bacterial count (PBC) and proteolytic psychrotrophic count (PPC) in different raw milk samples

Sample no	Counts per ml x 10^{-3}		
	TVC	PBC	PPC
1	72.00	11.00	2.70
2	26.00	3.20	1.95
3	7.00	4.50	1.85
4	11.00	4.00	1.00
5	5.80	2.10	0.43
6	6.50	0.40	0.20

Table 3.1.2 Distribution of psychrotrophic bacterial count (PBC) and proteolytic psychrotrophic count (PPC) in different raw milk samples

Sample no	% of TVC		
	PBC	PPC	PPC/PBC %
1	15.30	3.70	24.20
2	12.30	7.50	61.00
3	64.30	26.40	41.10
4	36.40	9.10	25.00
5	34.50	7.40	21.50
6	6.20	3.10	50.00

cultures were then transferred to nutrient agar (NA) slants and grown at 30°C for 48 h. These cultures were used for taxonomic tests.

3.1.4 Identification to the genus level

The morphological and biochemical tests proposed by Cowan & Steel (1974) were used to identify the unknown isolates. The following tests were carried out: Gram stain, cell shape, motility, oxidase reaction, catalase reaction, growth in air, anaerobic growth, oxidation/ fermentation and acid production from glucose. Tables 3.1.3 and 3.1.4 show the results of the tests used to identify the unknown strains to the genus level. A comparison of the results given in these tables and the diagnostic tables of Cowan & Steel (1974) and the tables given by Hendrie & Shewan (1979) lead to the conclusion that the Gram positive proteolytic psychrotrophic bacteria isolated belong to four genera: Bacillus, Micrococcus, Corynebacterium and Staphylococcus. The Gram negative bacteria were also assigned to one of four genera: Flavobacterium, Pseudomonas, Acintobacter and Cytophaga. The relative frequency of the isolates is shown in Table 3.1.5. Flavobacterium spp and Pseudomonas spp were the two main genera of Gram negative bacteria; they represented 48.8 and 34% respectively.

Malik & Mathur (1983) isolated 39 strains of highly active proteolytic psychrotrophs from raw milk, 41.7% were Pseudomonas spp, 17.9% Micrococcus spp, 15.4% each Flavobacterium spp and Alcaligenes spp and 10.2% Staphylococcus spp. In a similar study carried out by Richardson & Te

Table 3.1.3 Identification of Gram positive proteolytic psychrotrophic bacteria

Test performed	Isolate code			
	E	F	G	H
Cell shape	Sphere	Rod	Rod	Sphere
Motility	+	+	-	-
Oxidase reaction	+	+	-	-
Catalase reaction	+	+	+	+
Growth in air	+	+	+	+
Growth anaerobically	-	-	+	+
Oxidation/fermentation	Weak oxidative	Oxidative	Fermentative	Fermentative
Acid production from glucose	+	+	+	+

E = Z22 (Micrococcus spp)

F = Z11 (Bacillus spp)

G = Z6 (Corynebacterium spp)

H = Z29 (Staphylococcus spp)

Table 3.1.4 Identification of Gram negative proteolytic psychrotrophic bacteria

Test performed	Isolate code			
	A	B	C	D
Cell shape	Rod	Rod	Rod	Rod
Motility	-	+	-	-
Oxidase reaction	+	+	+	-
Catalase reaction	+	+	+	+
Growth in air	+	+	+	+
Growth anaerobically	-	-	-	-
Oxidation/fermentation	Fermentative	Oxidative	Oxidative	Oxidative
Acid production from glucose	+	+	+	+

A = R5, R19-R21, R23-R28, R30, R35-R39, R41-R43 (Flavobacterium spp)

B = R1-R4, R7-R10, R12-R16, R44 (Pseudomonas spp)

C = R31 and R34 (Cytophaga spp)

D = R40 (Acinetobacter spp)

Table 3.1.5 Identification of selected protease producing psychrotrophic isolates

Isolate code	Genus	Total number	%
A	Flavobacterium	20	48.8
B	Pseudomonas	14	34.0
C	Cytophaga	2	4.80
D	Acinetobacter	1	2.40
E	Micrococcus	1	2.40
F	Bacillus	1	2.40
G	Corynebacterium	1	2.40
H	Staphylococcus	1	2.40

A = Isolates R5, R19-R21, R23-R28, R30, R33, R35-R39 and R41-R43

B = R1-R4, R7-R10, R12-R16 and R44

C = R31 and R34

D = R40

E = Z22

F = Z11

G = Z6

H = Z29

Whaiti (1978), 67.5% of the 40 proteolytic psychrotrophs were Pseudomonads, 15% Staphylococci, 5% Aeromonads and a single species 2.5% each of Flavobacterium, Bacillus and Micrococcus. More than 90% of the protease-producing bacteria isolated by White et al. (1978) were of the genus Pseudomonas.

The majority of psychrotrophic Gram-negative rods isolated from raw milk are Pseudomonas spp (Richard, 1981; Mol & Vincentie, 1981; Malik & Mathur, 1983). In this study they are the second most common genus after Flavobacterium spp. The reason for that is two of the tested milk samples contained unusually high numbers of Flavobacterium spp (Plate 3.1.3). Milliere & Veillet-Poncet (1979) have shown that the Flavobacterium-Cytophaga group can represent up to 20% of the aerobic psychrotrophic population in refrigerated raw milk.

Fourteen isolates of Pseudomonas were chosen for further identification at the species level because most Pseudomonas spp are able to secrete extracellular proteases which can survive high temperature treatments and consequently cause spoilage, especially in sterilised dairy products (Fox, 1982; Patel et al., 1983; Patel et al., 1986; Mitchell et al., 1986).

3.1.5 Identification to species level

The identification of the 14isolatesof Pseudomonas spp was carried out using the Analytical Profile Index NE-20E (Section 2.7). Results shown in Table 3.1.6 indicate that Pseudomonas fluorescens was the dominant species (42.9%).

Two further tests were used, namely the flagella stain

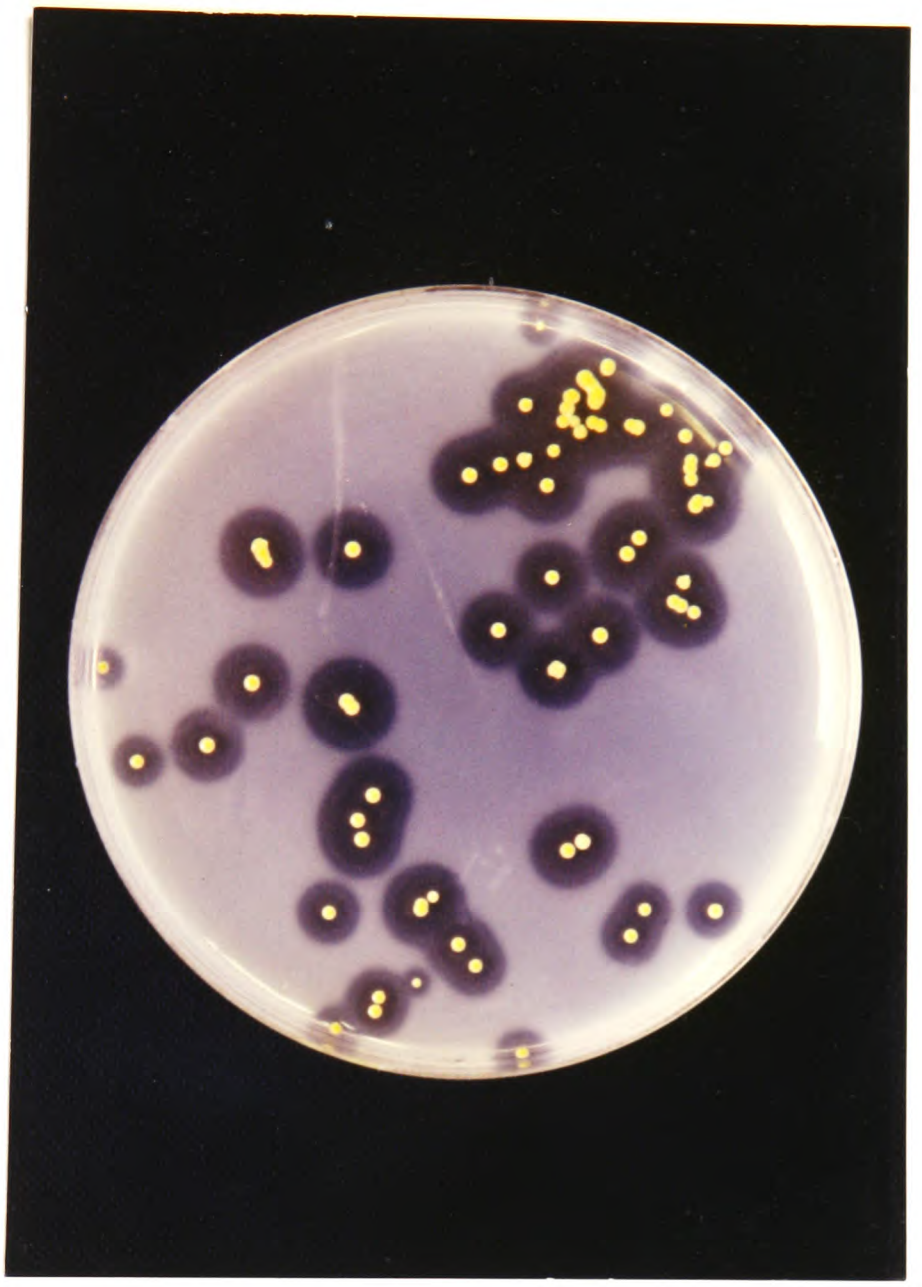


Plate 3.1.3. Proteolytic activity of Flavobacterium spp on SMA + 10% RSM

Incubation at 7°C for 10 d

Table 3.1.6 Distribution of Pseudomonas species isolated from raw milk

Species	Total number	%
Pseudomonas putida	3	21.40
Pseudomonas fluorescens	6	42.90
Pseudomonas aeruginosa	3	21.40
Pseudomonas vesicularis	1	7.10
Pseudomonas diminuta	1	7.10
	14	

and the production of fluorescent pigment. The position of flagella was examined using electron microscopy (Section 2.5.3). All the species studied showed polar flagella (Plate 3.1.4). Hendrie & Shewan (1979) in their studies on the differentiation of Pseudomonads from other Gram negative bacteria reported that the flagella of all Pseudomonads were polar whereas they were peritrichous for Flavobacterium.

The production of diffusible pigment was examined on the medium of King et al. (1954) (Section 2.6.8). All the isolates of Pseudomonas spp produced pigment. This is an iron binding siderophore (pyoverdine; Meyer & Hornsperger, 1978), which is produced by iron-deficient cells (Meyer & Abdallah, 1978).

Pseudomonas fluorescens strains R3, R8 and R44 showed more proteolytic activity than R2, R5, R15 when grown on SMA + 10% RSM. Plate 3.1.5 indicates that Pseudomonas fluorescens R8 showed higher proteolytic activity compared to Pseudomonas fluorescens R3 and Pseudomonas fluorescens R44 (the latter did not show a clear zone after incubation at 30°C for 2 d). P. fluorescens R8 was chosen for further studies on protease production (Section 3.2).

3.1.6 Summary

Eight genera of proteolytic psychrotrophic bacteria were isolated from raw milk. These genera were Flavobacterium spp, Pseudomonas spp, Acinetobacter spp, Cytophaga spp, Bacillus spp, Micrococcus spp, Corynebacterium spp and Staphylococcus spp. Pseudomonas fluorescens represented 42.9% of the Pseudomonas spp isolated. The

Plate 3.1.4. Polar flagellum of Pseudomonas fluorescens R12 (x 21 000)

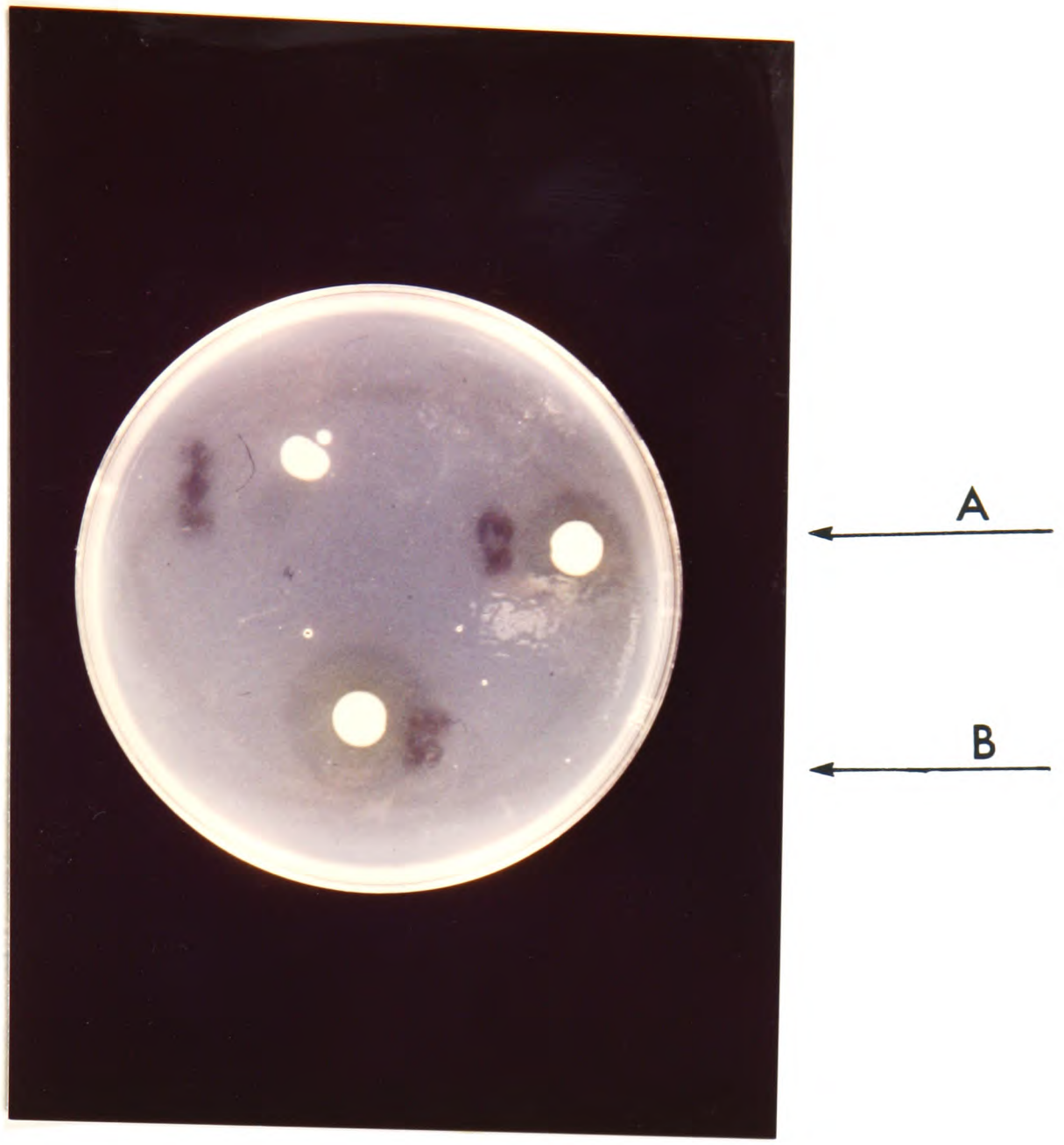


Plate 3.1.5. Zones of clearance due to proteolysis from (A) Pseudomonas fluorescens R3 (B) Pseudomonas fluorescens R8

Incubation at 30°C for 2 d on SMA + 10% RSM

most active proteolytic strain on SMA + 10% RSM was Pseudomonas fluorescens R8.

3.2 Growth and protease synthesis by Pseudomonas fluorescens R8

Preliminary experiments were carried out to compare between growth and enzyme production by three strains of P. fluorescens, namely R3, R8 and R44. The strains were grown in basal medium containing NH_4Cl and sodium pyruvate in the presence or absence of amino acids (Section 2.8.1). Growth was monitored by measuring the absorbance at 660 nm at appropriate time intervals. Enzyme assays using hide powder azure (HPA) as a substrate (Section 2.18.1) were determined in the supernatant of the growth medium after the strains approached the late exponential phase or the beginning of stationary phase. R8 was the most active strain in terms of enzyme production, whilst R44 produced a low amount of the enzyme. Enzyme produced per growth unit (EU mg dry weight^{-1}) was 0.04, 0.07 and 0.02 for R3, R8 and R44 respectively. These findings are in good agreement with the results obtained in Section 3.1.5 in which R8 showed the highest proteolytic activity on SMA + 10% RSM compared to R3 and R44. Therefore R8 was chosen for the investigation of the production of the protease.

3.2.1 Relationship between growth and biomass

The relationship between growth (A660) and biomass (mg dry weight ml^{-1}) was determined both for a complex medium (trypticase soy broth) and a minimal medium (mineral salts

medium with glutamate). Figures 3.2.1 and 3.2.2 show that there was a linear relationship between absorbance (A660) and dry weight between 0.2-0.9 absorbance units. The relationship between A660 and dry weight was similar for the basal and the complex media.

3.2.2 Effect of different energy sources

The effect of various energy sources on protease synthesis was determined using mineral salts medium (Section 2.8.1) supplemented with vitamins, NH_4Cl and different carbon sources. One flask was set up using mineral salts medium supplemented with glutamate as a sole source of carbon and nitrogen. Samples were taken of different stages during growth for protease assays (using azocasein as a substrate, Section 2.18.2); enzyme production was calculated as enzyme units mg dry weight ml^{-1}. Maximum level of the enzyme in all media (EU ml^{-1}) was observed when the culture approached late exponential phase or the beginning of stationary phase (Table 3.2.1). The same pattern was found when basal medium supplemented with sodium caseinate was used for production of the enzyme. The maximum production of the enzyme (EU $growth^{-1}$) was also found at the end of exponential phase or at the beginning of stationary phase in both glucose basal medium and in the basal medium supplemented with glutamate as a sole source of carbon and nitrogen. This activity remained constant until the end of the stationary phase. The enzyme produced in these two media was about 2-4 fold higher than the enzyme produced with other carbon sources (Table 3.2.1). Basal medium supplemented with glutamate and glucose (sole

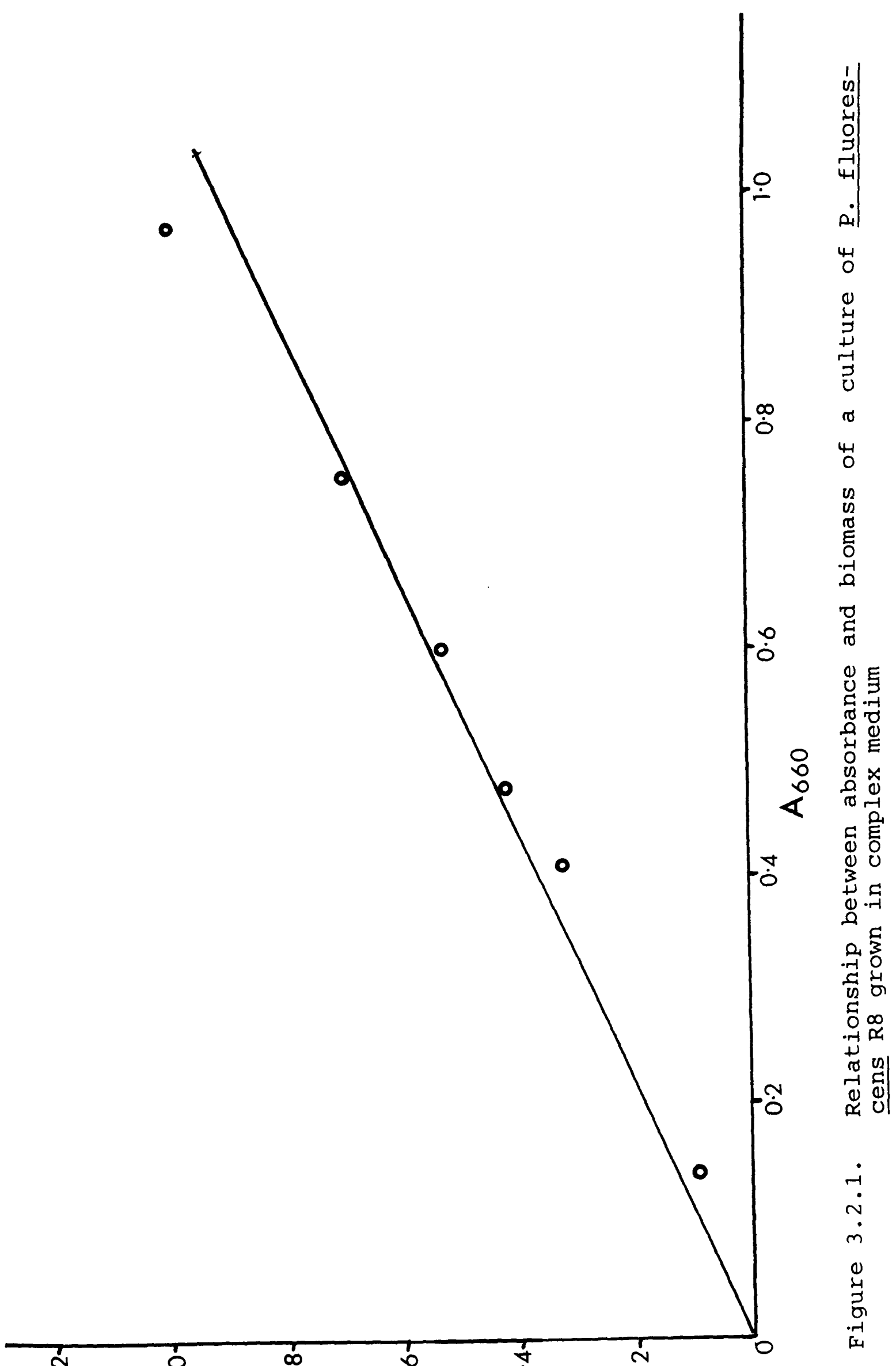


Figure 3.2.1. Relationship between absorbance and biomass of a culture of P. fluorescens R8 grown in complex medium

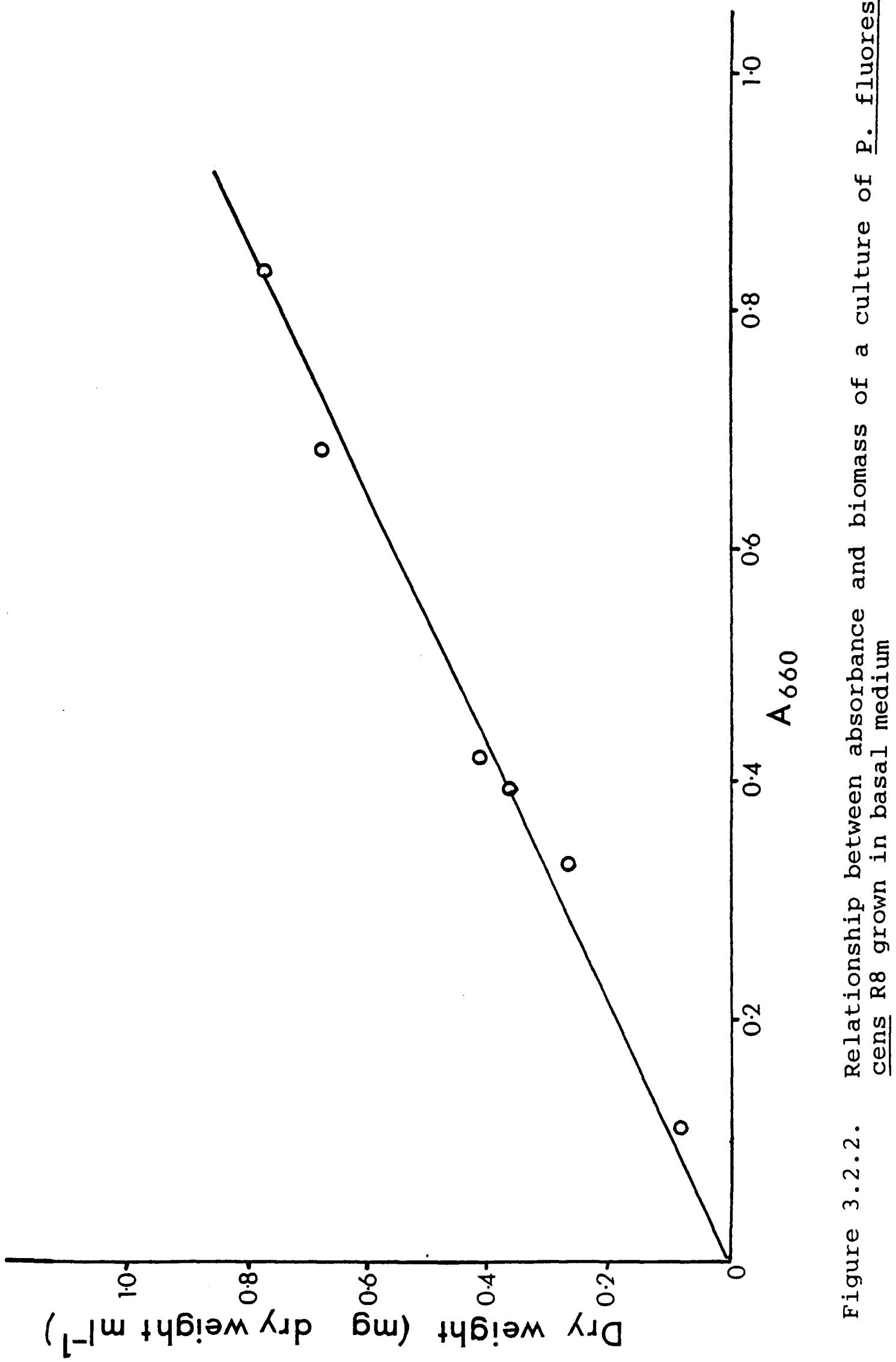


Figure 3.2.2. Relationship between absorbance and biomass of a culture of P. fluorescens R8 grown in basal medium

Table 3.2.1 Production of protease by Pseudomonas fluorescens R8 in basal medium supplemented with various carbon sources

Carbon source	Concentration (mM)	Growth (mg dry weight ml^{-1})	Enzyme units $(min^{-1} ml^{-1})$	EU growth^{-1} (EU mg dry weight^{-1})
Succinate	40	0.94	0.08	0.09
Malate	40	0.63	0.03	0.05
Glucose	40	1.24	0.22	0.18
Pyruvate	20	0.39	0.03	0.08
Pyruvate	40	0.69	0.06	0.09
Aspartate	40	1.24	0.09	0.07
Glutamate	40	1.13	0.21	0.19

Growth and enzyme activity was measured when the bacteria reached the beginning of stationary phase

source of carbon and nitrogen) were chosen for studying the production of the protease in detail (Sections 3.2.3 and 3.2.5).

3.2.3 Enzyme activity during growth on complex and basal media

Enzyme activity during growth was compared for growth on complex medium with trypticase soy broth (TSB) as a source of proteins and amino acids and for growth on basal medium with glutamate as a sole source of carbon and nitrogen (Figures 3.2.3 and 3.2.4). On TSB medium, Pseudomonas fluorescens R8 approached the end of exponential phase after ≈ 16 h of growth. Maximum production of the enzyme (EU growth^{-1}) was found to be at the end of the exponential phase and beginning of stationary phase. Maximum activity per growth was 0.52 (EU mg dry weight^{-1}). However, when glutamate basal medium was used as a sole source of carbon and nitrogen R8 reached the stationary phase after ≈ 112 h and the maximum activity per growth was 0.20 and 0.14 in the presence and absence of $CaCl_2$ respectively (Figures 3.2.4 and 3.2.5). The principal nitrogenous components of TSB are products from a pancreatic digest of casein and these products might be expected to act as inducers for the protease. However, the activity found in TSB medium was only about 2.6 fold higher than that found in glutamate basal medium with the presence of $CaCl_2$. It could be concluded from these results that P. fluorescens R8 produced a constitutive protease, whose production was not highly affected by the presence of inducers.

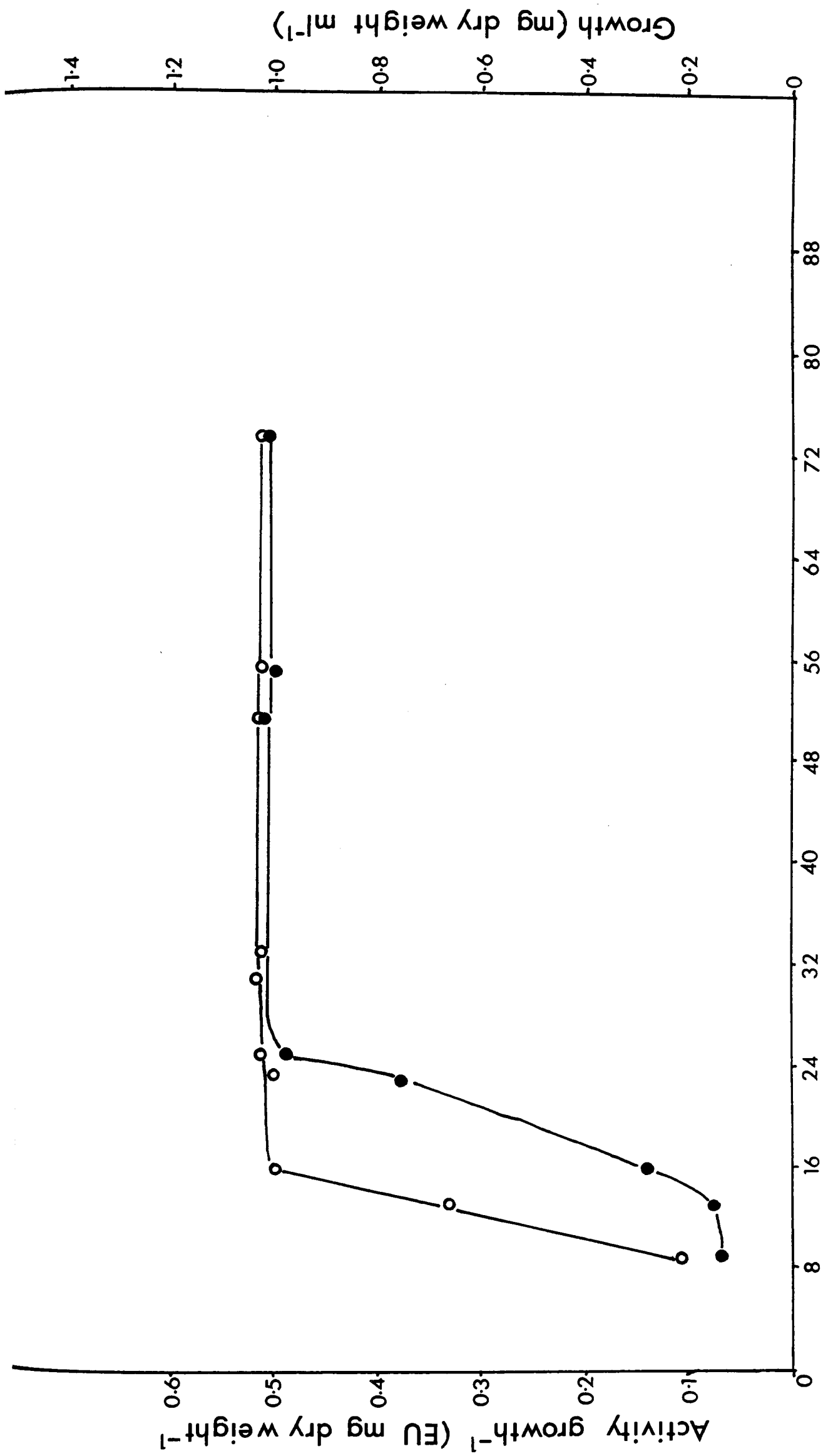


Figure 3.2.3. Production of protease by P. fluorescens R8 in complex medium (TSB)

○—○ Growth mg dry weight ml^{-1}

●—● Activity growth^{-1} (EU mg dry weight^{-1})

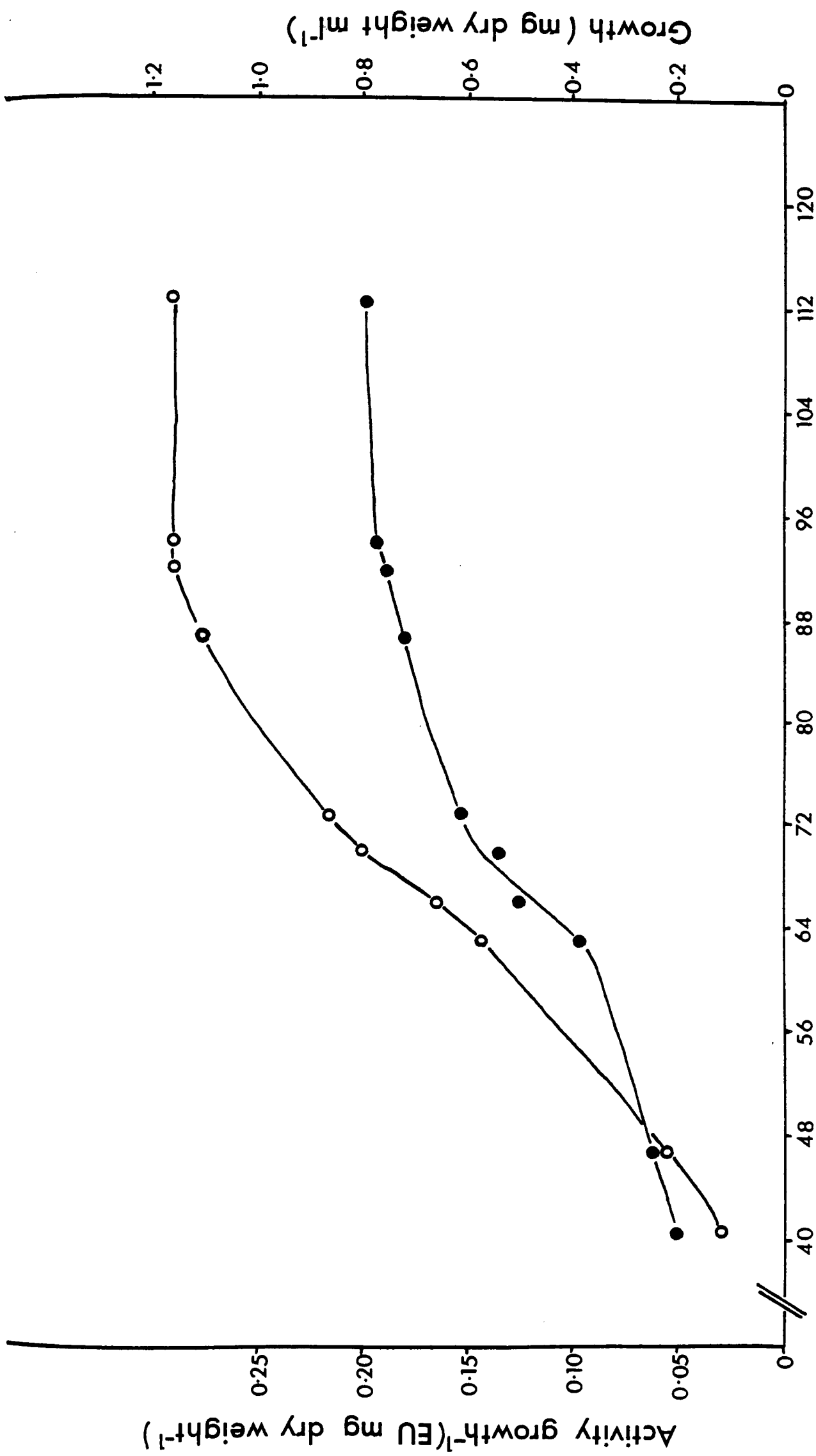


Figure 3.2.4. Growth and protease production in glutamate basal medium in the presence of $CaCl_2$

o——o Growth (mg dry weight ml^{-1})

●——● Activity growth^{-1} (EU mg dry weight^{-1})

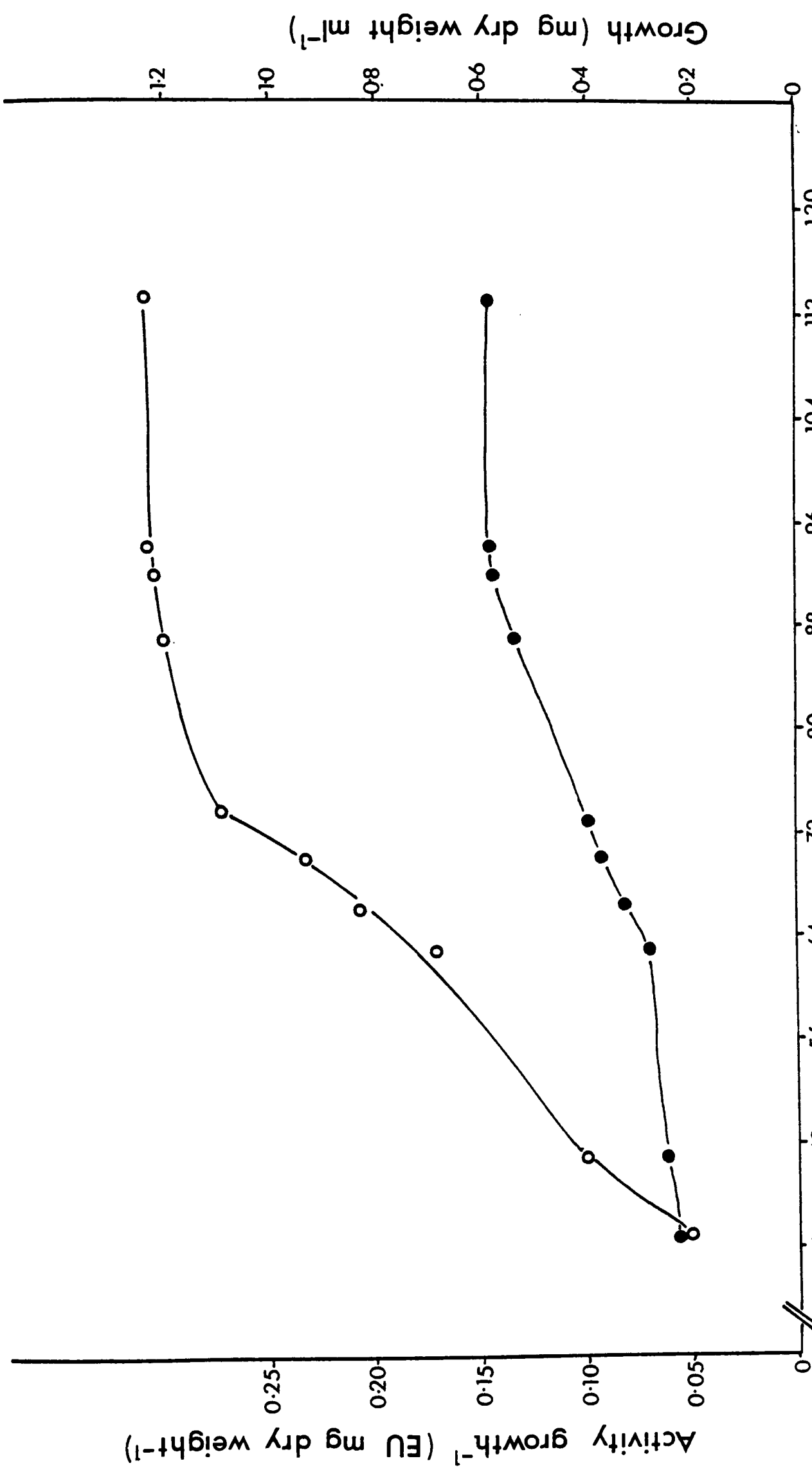


Figure 3.2.5. Growth and protease production in glutamate basal medium in the absence of $CaCl_2$

o—o Growth (mg dry weight ml^{-1})

●—● Activity growth^{-1} (EU mg dry weight^{-1})

3.2.4 Quantitation of protease protein using rocket immunoelectrophoresis

Rocket immunoelectrophoresis (RIE) constitutes a simple method for quantitating protein based on the cross reaction between antigen (protease) and antibodies raised against the enzyme. Preliminary studies showed that the quality of the antibodies raised against the enzyme when mice were used was better than using rabbit antibodies. In order to quantitate protease protein, a standard curve was constructed. The construction of a standard curve was achieved by loading increasing amounts of enzyme protein in a fixed volume onto the gels and measuring the height of the precipitate (peak). Plots of rocket height against amount of protein loaded revealed linear relationships only with loadings in the range 45-233 ng purified enzyme. Above or below these figures the relationship was not linear. Some other problems were also encountered such as precipitation around the wells and a decrease in sharpness at the rocket tips.

It was also necessary to determine the optimum anti-serum concentration in order to optimise conditions for the RIE standard curve. Increasing amounts of antiserum were added to 3.3 ml of agarose and RIE was carried out as described in Section 2.19.3. Diluted antiserum (25 ml 1:0.05) yielded a measurable range of rocket heights. The highest of the rocket peaks was measured and plotted against the amount of enzyme loaded. Figure 3.2.6 shows the results obtained by this technique and Plate 3.2.1 shows the rocket immunoelectrophoresis gel of the purified

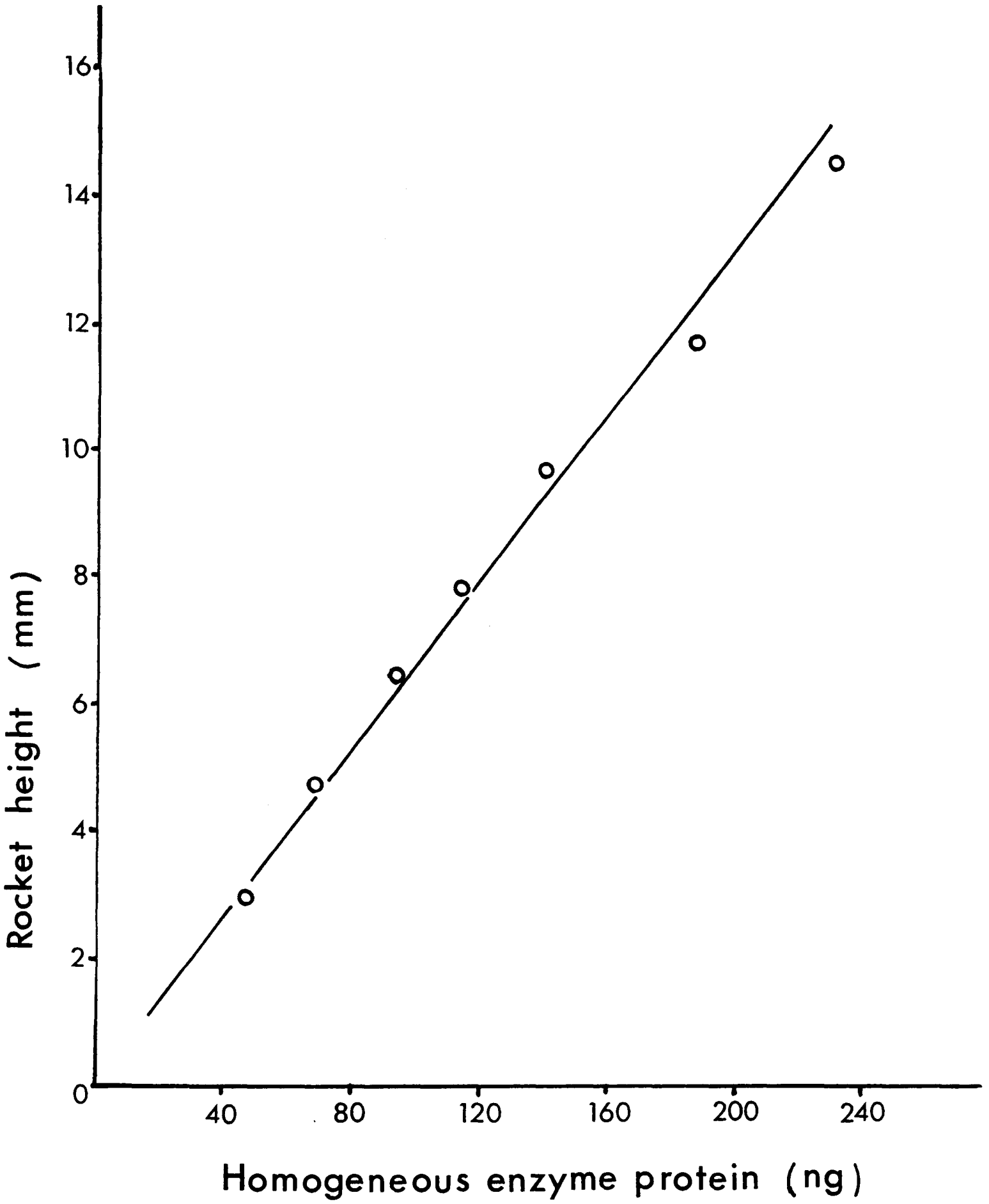


Figure 3.2.6. Rocket heights of immunoprecipitates after rocket immunoelectrophoresis of the homogenous protease

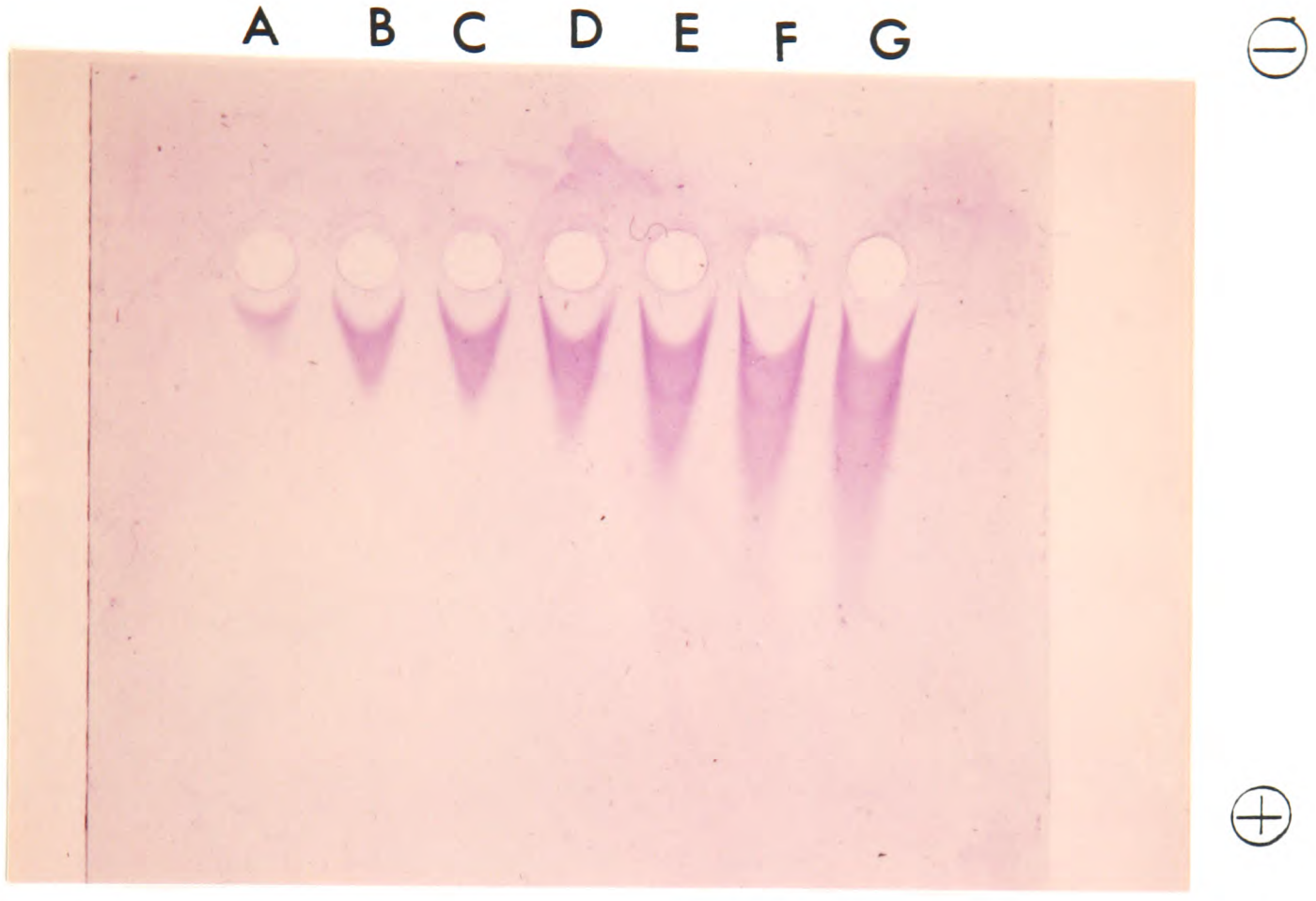


Plate 3.2.1. Rocket immunoelectrophoresis gel of the purified protease.

The protein loadings per well (ng protein) were as follows:

A - 46

B - 68

C - 94

D - 116

E - 142

F - 188

G - 233

protease which was used to construct the calibration curve.

3.2.5 Enzyme activity and synthesis in glucose basal medium

Rocket immunoelectrophoresis was used to monitor the synthesis of protease protein during growth in glucose basal medium in the presence and absence of $CaCl_2$; the bacteria reached the beginning of stationary phase after ≈ 45 h. No difference in growth was noticed when $CaCl_2$ was omitted from the medium. However, the enzyme produced in the presence of $CaCl_2$ was about seven times higher than in the absence of $CaCl_2$ when activity per growth was measured at the end of exponential phase (Figures 3.2.7 and 3.2.8). Enzyme protein was detected both in the presence and absence of $CaCl_2$ using RIE technique (Plates 3.2.2 and 3.2.3). It was found that the enzyme protein concentration in the presence of $CaCl_2$ was about 10 fold higher than in the absence of $CaCl_2$ when the bacteria approached the end of exponential phase. The synthesis of enzyme protein (µg enzyme protein growth^{-1}) remained almost constant throughout the growth cycle either in the presence or absence of $CaCl_2$. However there was apparent activation of the enzyme when $CaCl_2$ was present in the medium. In glucose basal medium containing $CaCl_2$, activity per growth increased about 4.3 fold through the growth cycle and the specific activity (EU µg enzyme protein^{-1}) increased about 6 fold. No significant increase in specific activity was found when $CaCl_2$ was omitted from the medium.

The enzyme was synthesised at the early stages of growth and was detected in the glucose basal medium in the

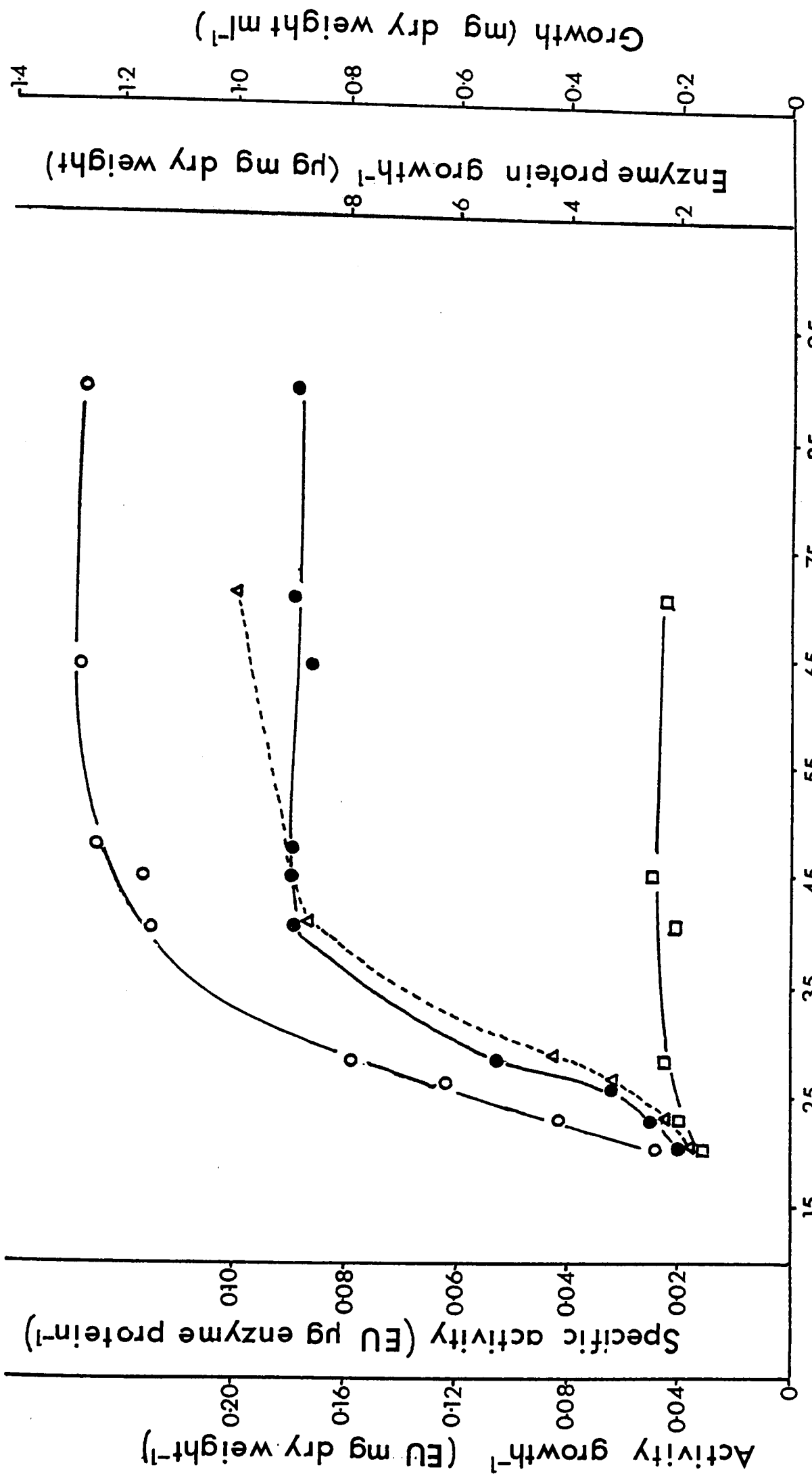


Figure 3.2.7. Growth and production of protease in glucose basal medium in the presence of $CaCl_2$

O—O Growth (mg dry weight ml^{-1})

●—● Activity $growth^{-1}$ (EU mg dry $weight^{-1}$)

△—△ Specific activity (EU µg enzyme $protein^{-1}$)

□—□ Enzyme protein $growth^{-1}$ (µg enzyme protein $growth^{-1}$)

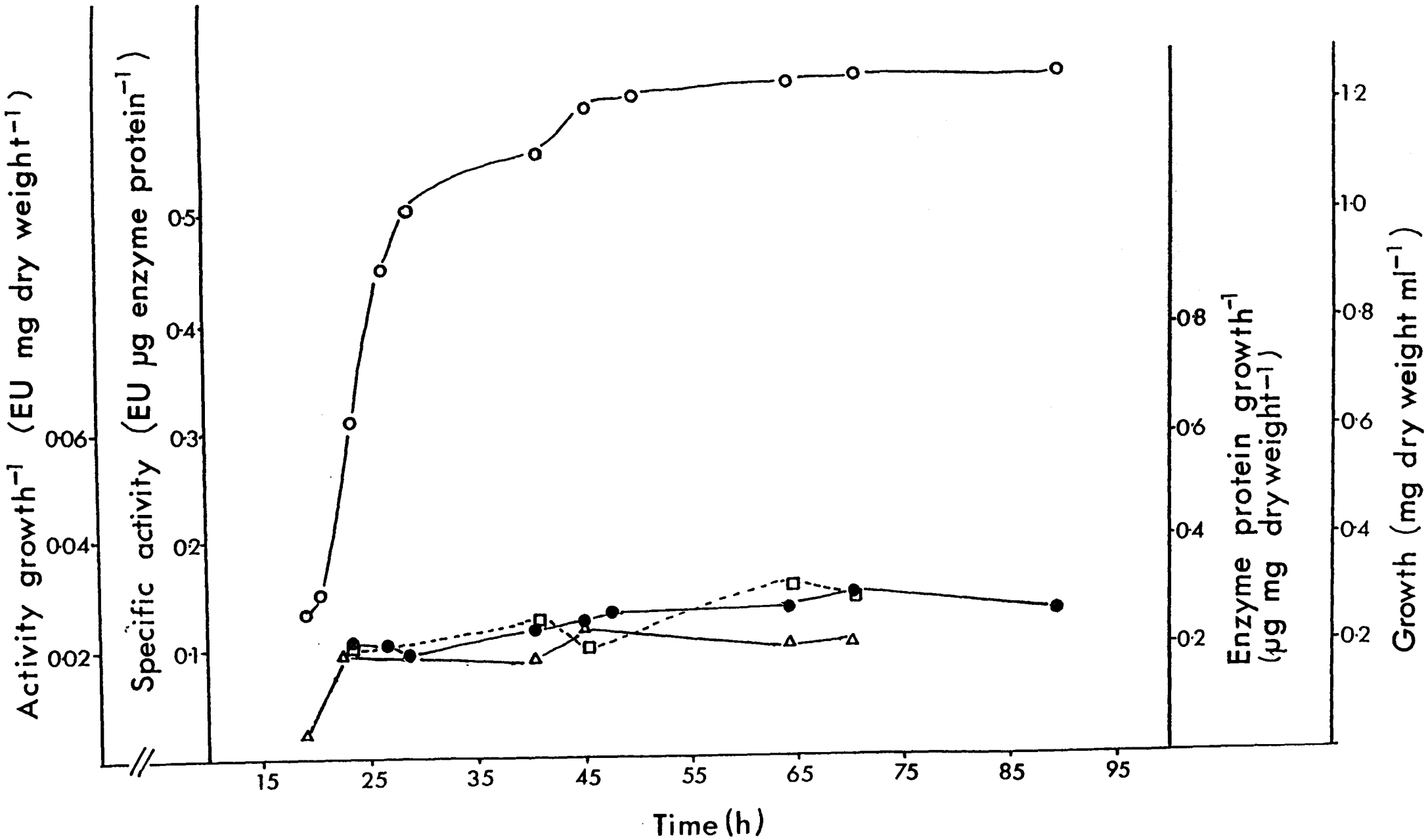


Figure 3.2.8. Growth and production of protease in glucose basal medium in the absence of $CaCl_2$

o——o Growth (mg dry weight ml^{-1})

●——● Activity $growth^{-1}$ (EU mg dry $weight^{-1}$)

□——□ Specific activity (EU µg enzyme $protein^{-1}$)

△——△ Enzyme protein $growth^{-1}$ (µg enzyme protein $growth^{-1}$)

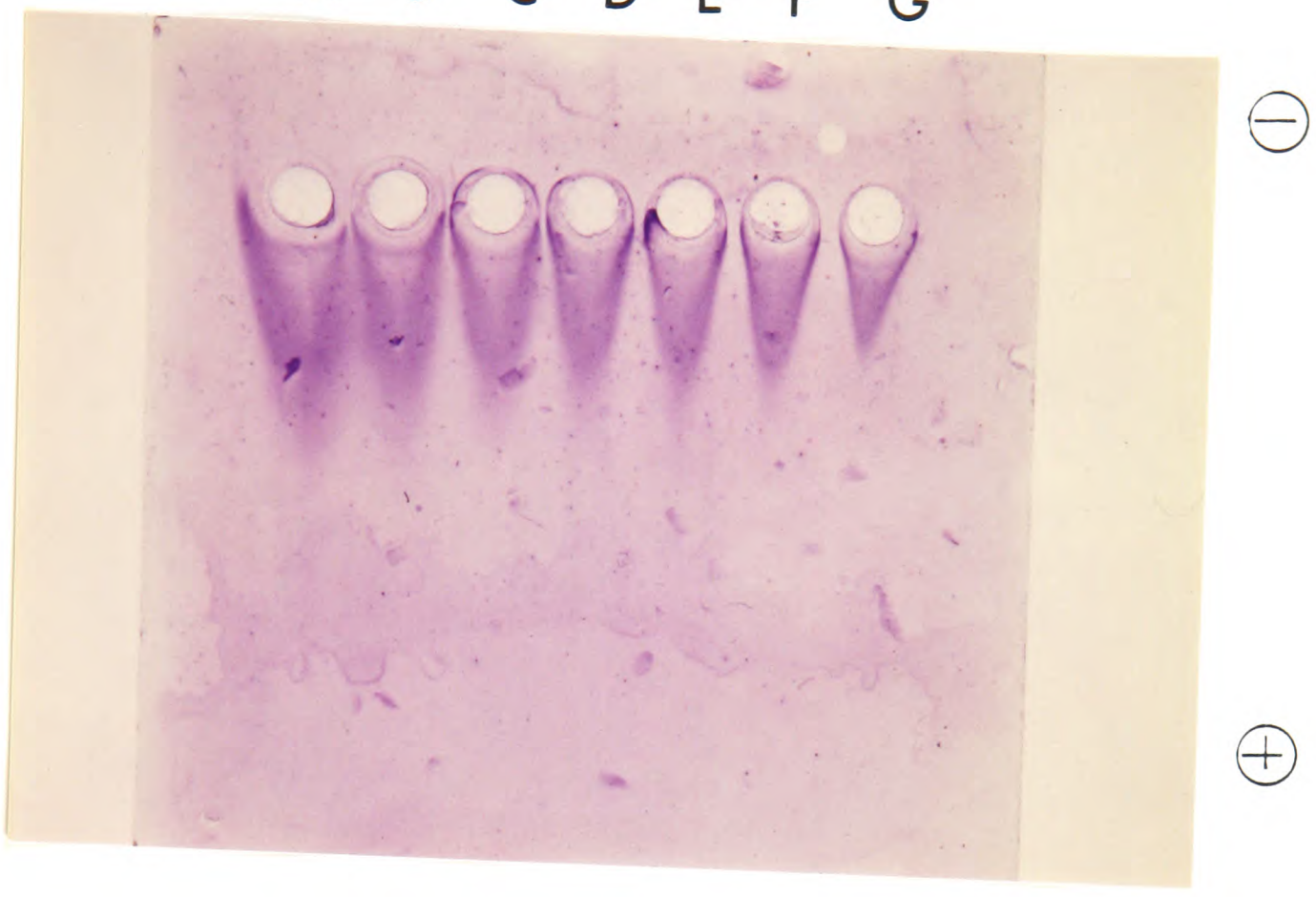


Plate 3.2.2. Rocket immunoelectrophoresis gel of the protease synthesised in glucose basal medium in the presence of $CaCl_2$.

The protein loadings per well (ng protein) were as follows:

A - 1170

B - 585

C - 390

D - 334

E - 293

F - 234

G - 195

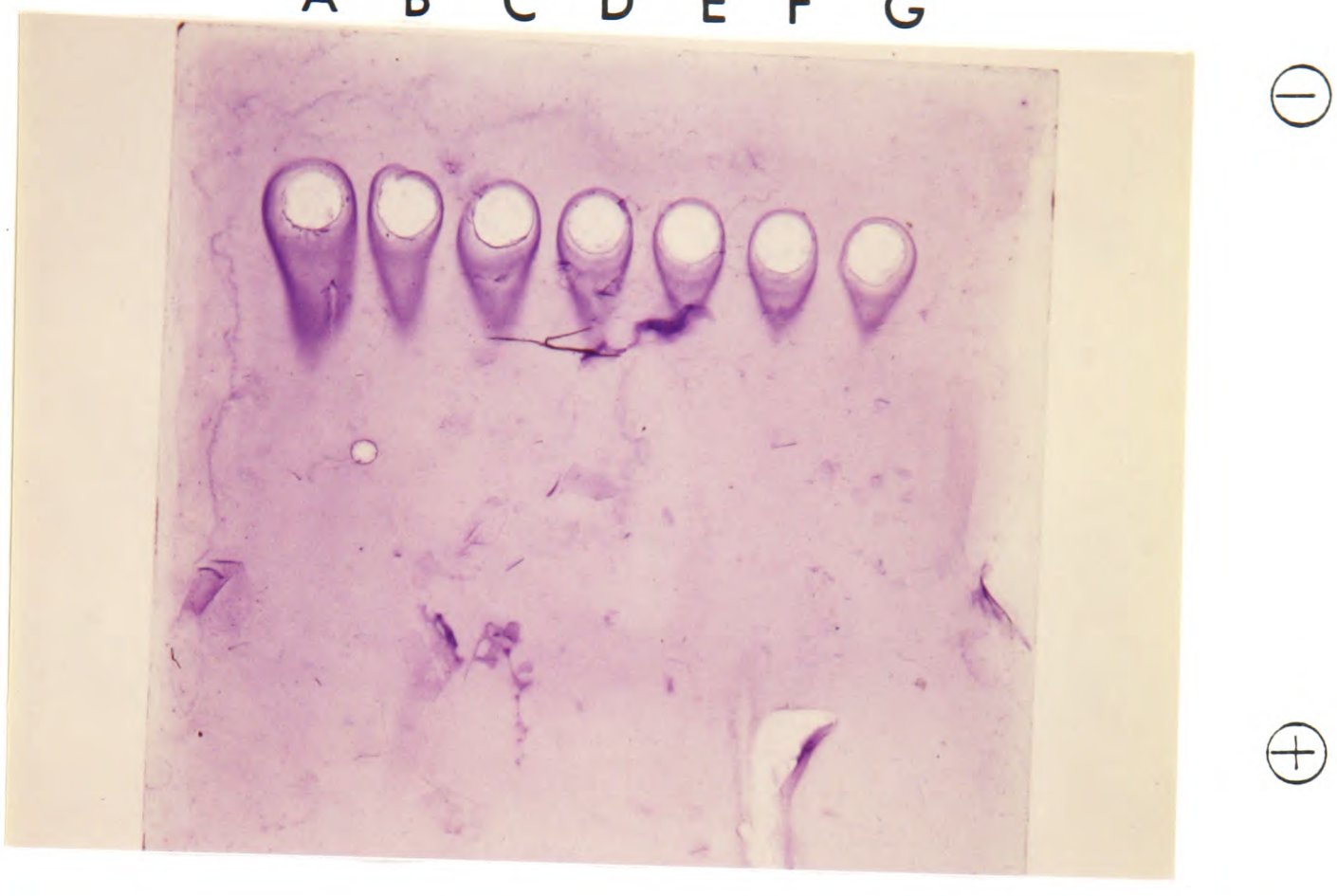


Plate 3.2.3. Rocket immunoelectrophoresis gel of the protease synthesised in glucose basal medium in the absence of $CaCl_2$.

The protein loadings per well (ng protein) were as follows:

A - 5580

B - 3720

C - 2790

D - 1860

E - 1395

F - 1116

G - 797

absence of $CaCl_2$ (Plate 3.2.4).

3.2.6 Discussion

The results obtained in this study demonstrate that Pseudomonas fluorescens R8 is able to grow and synthesise protease in both basal and complex media. The strain utilised glutamate as a sole source of carbon and nitrogen and produced protease when grown in this medium. The amount of protease secreted (activity $growth^{-1}$) in complex medium was between 2.6-2.8 fold higher compared to the enzyme produced in basal medium with either glucose or glutamate in the presence of $CaCl_2$. These results led to the conclusion that strain R8 may be considered to synthesise a constitutive protease as protease synthesis was not greatly affected by the presence of proteins or protein degradation products in the medium. In this respect it resembles the plant pathogen Pseudomonas lachrymans in which protease production was not significantly affected by the protein source in the growth medium (Keen & Williams, 1967). Boethling (1975) also found that Pseudomonas maltophilia produced a constitutive protease, synthesised maximally in the presence or absence of substrate or derivatives of the substrate. Some extracellular enzymes are called semi-constitutive; that means they are present at low level, but their presence can be highly stimulated by inducers (Thomas et al., 1980). On the other hand Pseudomonas fluorescens NCDO2085 did not produce an extracellular protease when grown in the presence of inorganic nitrogen (Fairbairn & Law, 1987). The enzyme produced by this strain was considered to be inducible.

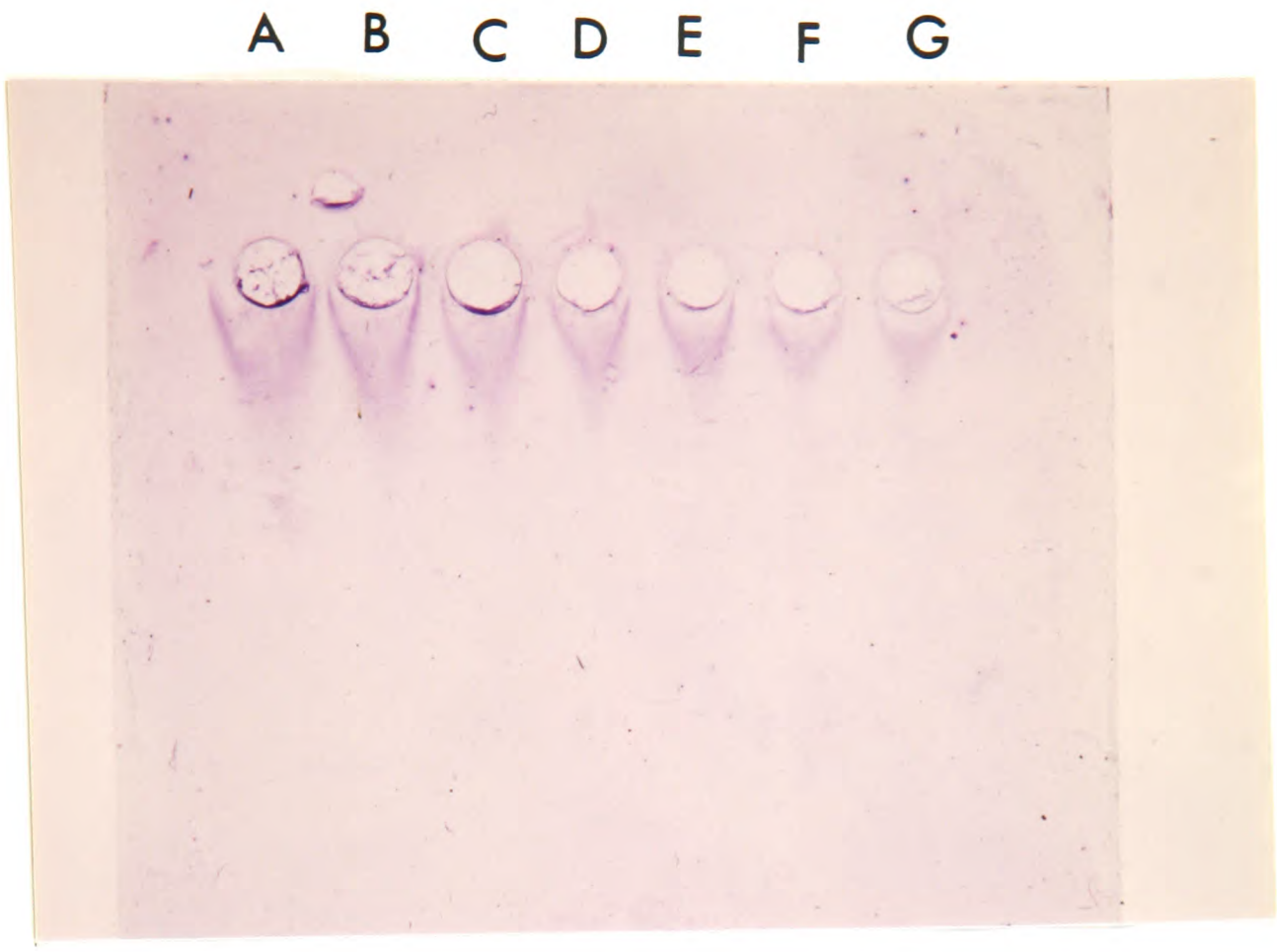


Plate 3.2.4. Rocket immunoelectrophoresis gel of the protease synthesised in glucose basal medium at the beginning of growth in the absence of $CaCl_2$

The protein loadings per well (ng protein) were as follows:

A - 4320

B - 2880

C - 2160

D - 1440

E - 1080

F - 864

G - 720

Strain R8 grew and produced enzyme in basal medium supplemented with different carbon sources. The strain grew faster in glucose, aspartate and glutamate basal media compared to other carbon sources. However, there was no significant difference between the enzyme produced in glutamate basal medium and glucose basal medium when $CaCl_2$ was present in the media. Fairbairn & Law (1987) reported that glucose was a poor carbon source for Pseudomonas spp which lack the glycolytic pathway, and was also a poor repressor of protease production. They also found that citrate was utilised as an energy source but had a strong repressive effect on protease production.

The effect of $CaCl_2$ on the production of the protease was studied. The presence of $CaCl_2$ in glutamate basal medium only slightly increased the enzyme produced per mg dry weight whereas in glucose basal medium the presence of $CaCl_2$ increased production of the enzyme about 7 fold compared to its absence. Stimulation of apparent protease production by calcium has been previously reported by Amrute & Corpe (1978) and Fairbairn & Law (1987) for P. fluorescens. Amrute & Corpe (1978) found a 9 fold increase for glucose-alanine basal medium and Fairbairn & Law (1987) a 5 fold increase for asparagine minimal medium. McKellar & Cholette (1986) revealed that in the absence of $CaCl_2$ in the growth medium of P. fluorescens B52, a low molecular weight (12-14 K daltons) irreversibly inactive precursor was formed. Other cations such as Fe^{2+}, Zn^{2+} and Mg^{2+} were found to affect either the production or the activity of proteases secreted by some strains of Gram-positive and

Gram-negative bacteria (Bjorn et al., 1979).

Maximum amount of protease (activity growth^{-1}) synthesised by strain R8 was detected at the late exponential phase and beginning of stationary phase in both complex and basal medium. However protease production was detectable right from the beginning of the exponential phase. Previous reports have been contradictory. On the one hand the pattern of extracellular protease produced by Pseudomonas spp was found to be either in the late logarithmic phase or early stationary phase (Juan & Cazzulo, 1976; Whooley et al., 1983; Fairbairn & Law, 1987). On the other hand McKellar & Cholette (1986) and Amrute & Corpe (1978) reported that optimum production of the protease by P. fluorescens was found to be at the mid-logarithmic phase.

The synthesis of protease protein was studied by rocket immunoelectrophoresis. Measurement of protein synthesis rather than enzyme activity was very sensitive and enzyme protein could be detected down to 45 ng. The enzyme protein increased about 2.4 fold throughout the growth cycle when strain R8 was grown in glucose basal medium in the absence of $CaCl_2$. However, in the presence of $CaCl_2$ the increase was about 6.2 fold indicating that calcium ions may affect the synthesis of protease. It has also been found that in the presence of $CaCl_2$ the amount of enzyme protein was about 10 fold higher than in the absence of $CaCl_2$. Specific activity (EU µg enzyme protein^{-1}) was almost the same throughout growth in the absence of $CaCl_2$ but increased about 6 fold in the presence of $CaCl_2$.

CHAPTER 4

PURIFICATION AND CHARACTERISATION OF PROTEASE

4.1 Purification of the protease

4.1.1 Ultrafiltration and ammonium sulphate precipitation of culture supernatant

Preparation of protease extract was carried out as described in Section 2.10. Ultrafiltration was chosen for the first stage of purification because of the large volume of culture supernatant. This technique was satisfactory as within 30 min the culture supernatant was concentrated approximately 5-fold from 5.0 l to c 1.0 l. In the ultrafiltration step the protease was purified 1.33-fold with 87% recovery.

In the second stage of purification the concentrated enzyme was precipitated by ammonium sulphate fractionation. Precipitation was performed according to the procedures described in Section 2.13. The protein concentration, specific and total activities of the protease obtained at each stage of precipitation are shown in Figures 4.1.1, 4.1.2 and 4.1.3. The results from the ammonium sulphate fractionation indicated that about 70% of the total activity was present in the 40-60% pellet. A 2-fold purification was achieved by this step (Table 4.1.1).

Native-PAGE (Section 2.16.1) and zymogram staining (Section 2.17) were carried out to monitor the progress of purification and to locate the enzyme on gels. The protein profile from 40-60% $(NH_4)_2SO_4$ precipitate was different from that seen with concentrated crude enzyme (Plate 4.1.1). Some protein bands disappeared after $(NH_4)_2SO_4$ fractionation as a result of purification. On the other hand, new bands were visualised. The appearance of new

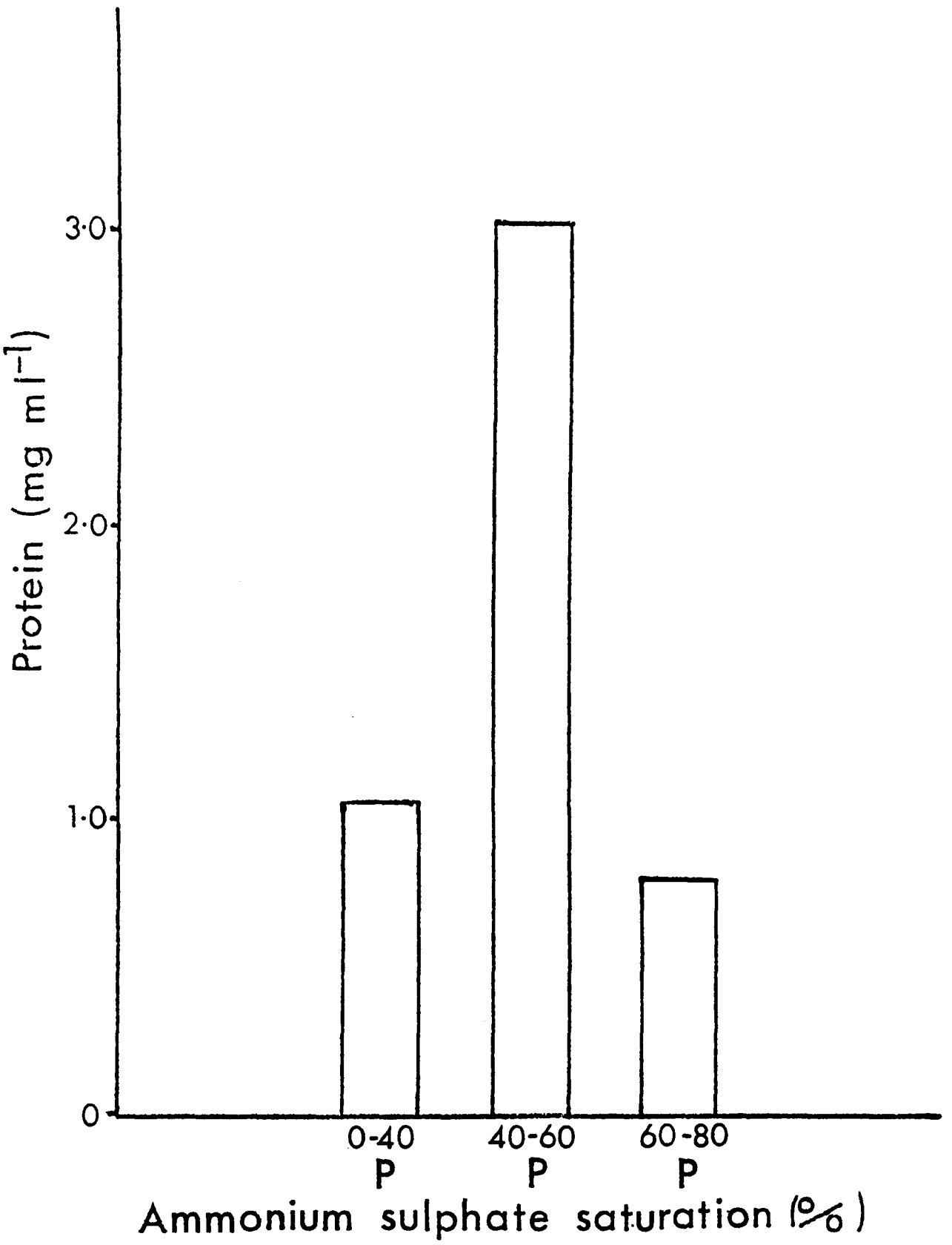


Figure 4.1.1. Precipitation of concentrated culture supernatant by ammonium sulphate

P - precipitate

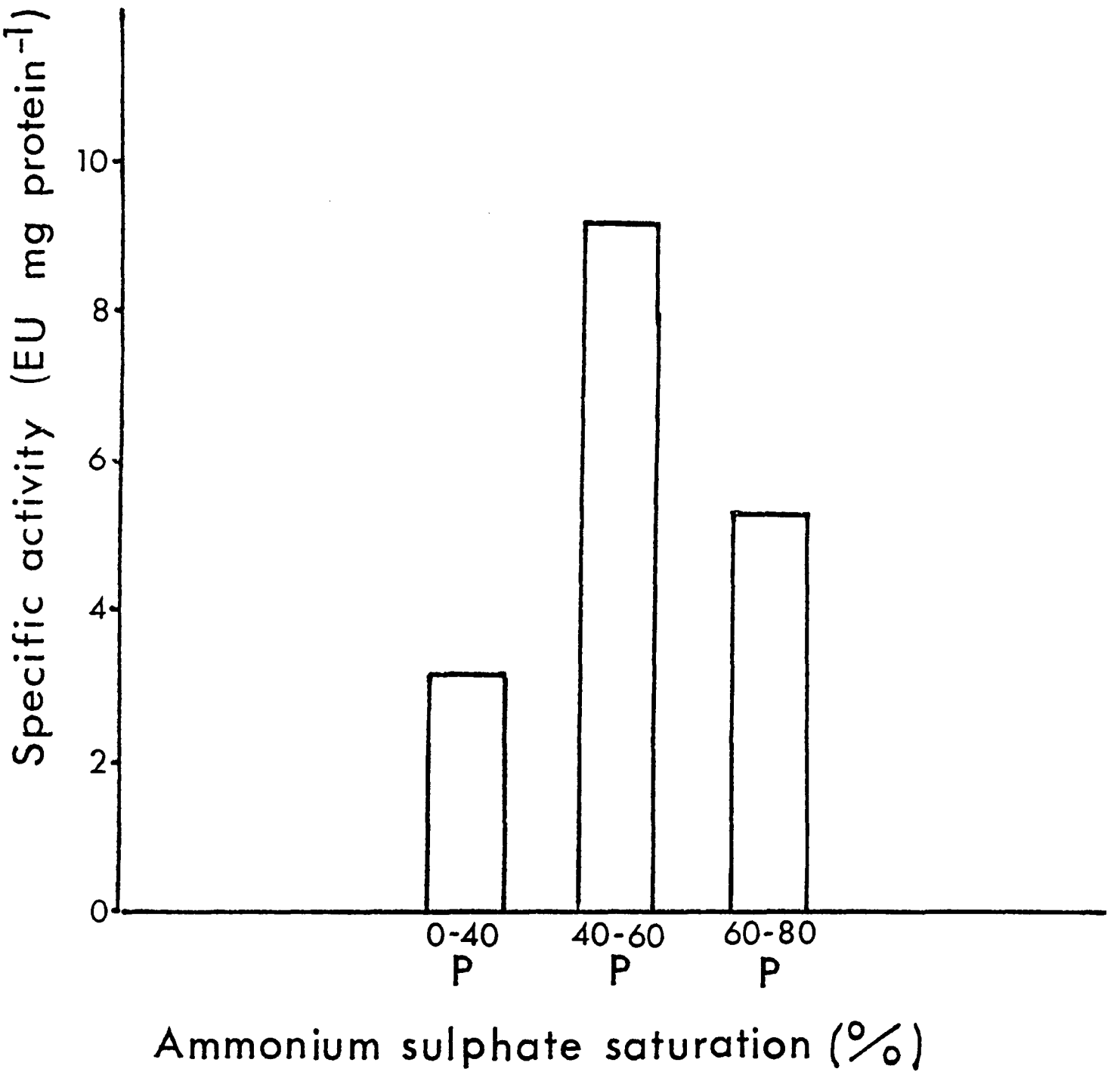


Figure 4.1.2. Effect of ammonium sulphate fractionation on specific activity in concentrated culture supernatant

P - precipitate

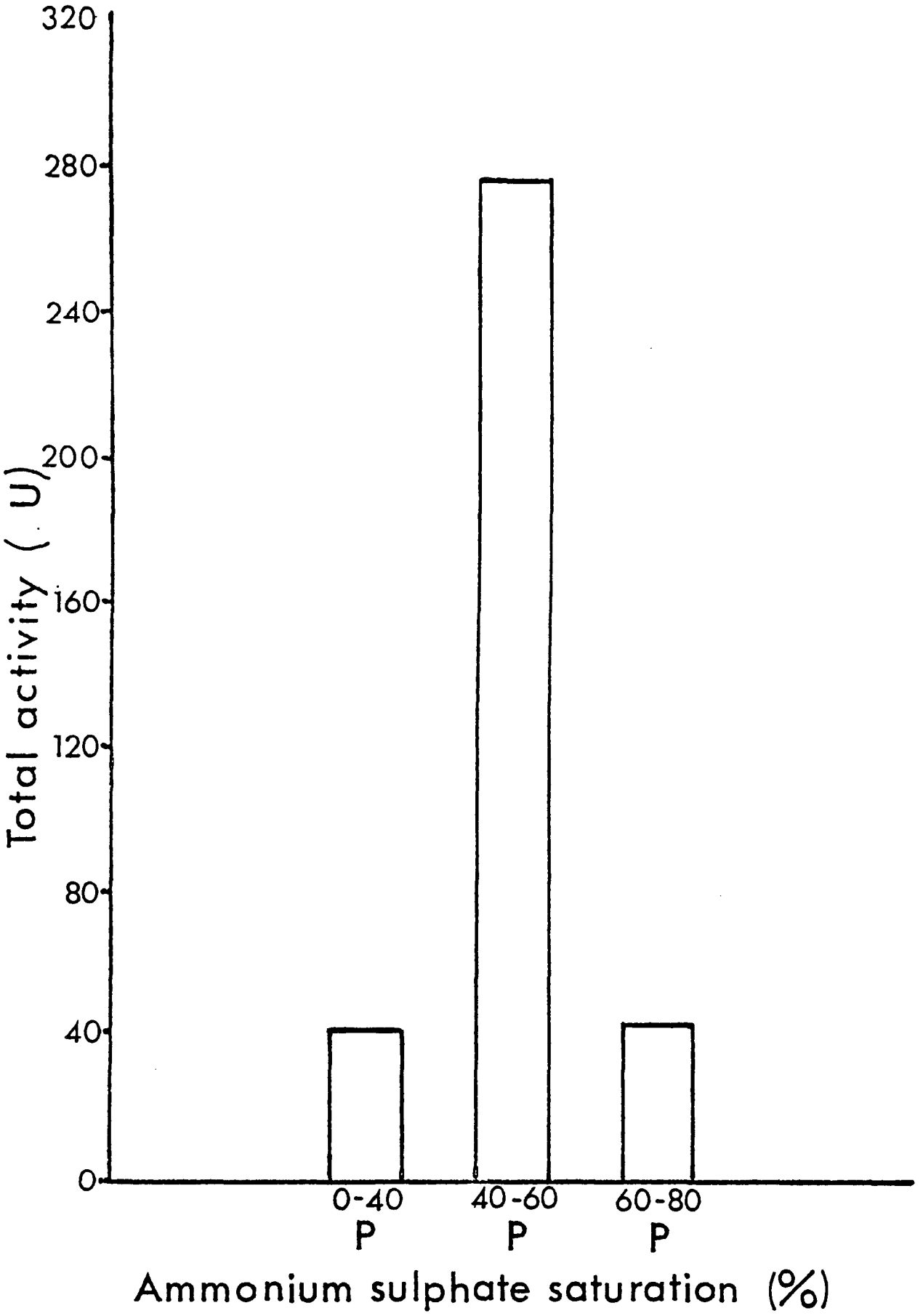


Figure 4.1.3. Effect of ammonium sulphate fractionation on total activity in concentrated culture supernatant

P - precipitate

Table 4.1.1 Effect of ammonium sulphate precipitation on the protease activity

$(NH_4)_2SO_4$ (%)	Purification[1] (fold)	Yield[2] (%)
Precipitate (0-40)	0.84	10
Supernatant (0 -40)	1.13	63.5
Precipitate (40-60)	2.00	68.4
Supernatant (40-60)	0.29	3.2
Precipitate (60-80)	1.14	10.2
Supernatant (60-80)	0.18	0.5

[1] Increase in protease specific activity

[2] Total recovered activity

Plate 4.1.1. PAGE of the concentrated supernatant and 40-60% $(NH_4)_2SO_4$ precipitate.

		µg loaded
A)	40-60% precipitate	20
B)	Concentrated crude enzyme	20

(7.5-15% acrylamide, silver stain)

protein bands was probably due to autolysis during electrophoresis rather than concentration of previously minor bands. The evidence for this is given in Section 4.1.3. When a zymogram stain was performed to detect the enzyme protein no distinct clear zones were seen. Instead a smeary clear zone was observed. In contrast to this a distinct clear zone was seen when Bacillus polymxa protease was used as a standard protease (Plate 4.1.2.). The smeary clear zone started from the stacking gel down to about two-thirds of the resolving gel (Plates 4.1.2 and 4.1.3). The same feature was seen when the native gel was placed on the surface of a protease detection medium (Section 2.17). Due to the appearance of the smeary clear zone it was difficult to locate the enzyme on the gel. The appearance of a smeared indistinct clear zone was probably due to aggregation of the enzyme on the top of the gel. This was later confirmed (see Sections 4.1.3 and Plate 4.1.7).

Several trials were done in attempts to improve the zymogram stain. These included using ovalbumin and azocasein as alternative substrates and changing the soaking time. None of the variations improved the background or increased the amount of substrate bound to the gel.

In order to overcome the problem of aggregation SDS-PAGE was carried out. Boiling the enzyme in the presence of SDS followed by electrophoresis in the presence of SDS disaggregates protein complexes and breaks down oligomeric proteins into their constituent subunits. However even on SDS-PAGE there was inconsistency in the number of bands obtained with the active enzyme in ammonium sulphate

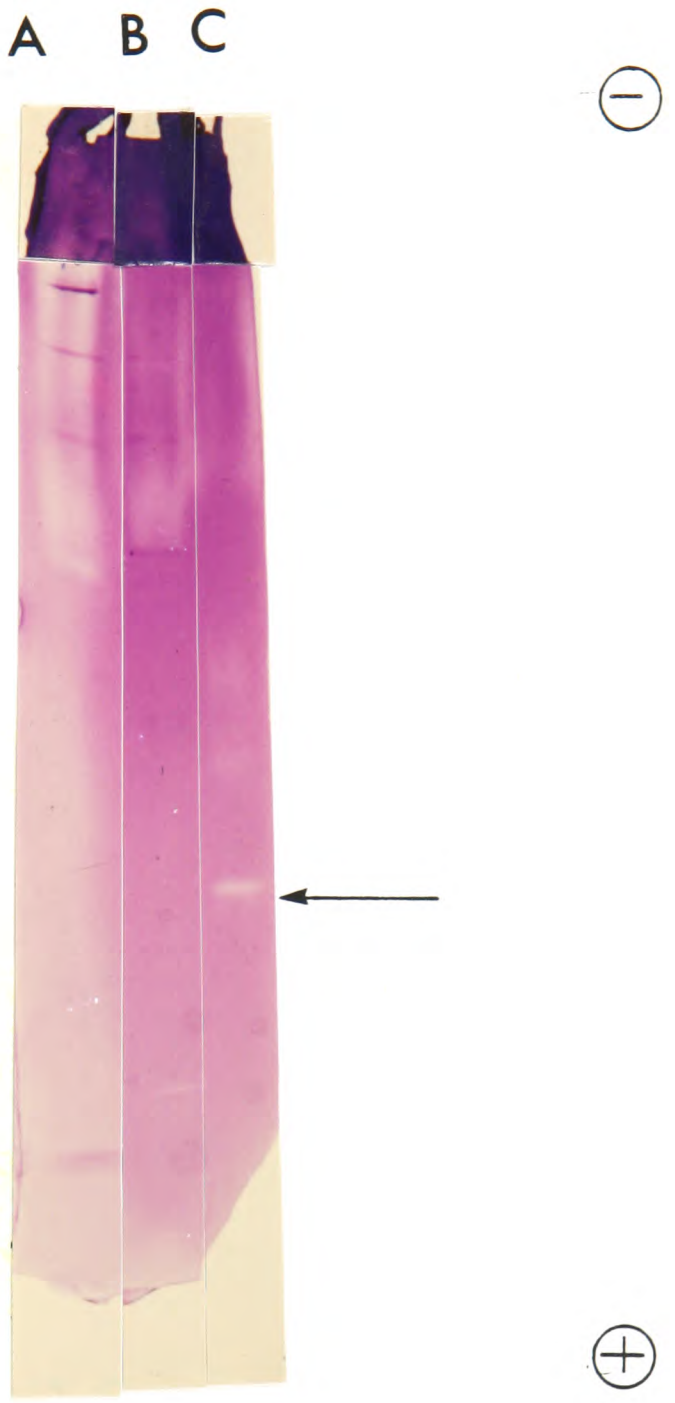


Plate 4.1.2. Native-PAGE (zymogram stain)

Sample		µg loaded
A)	Concentrated crude enzyme	50
B)	40-60% $(NH_4)_2SO_4$ precipitate	50
C)	Protease from <u>Bacillus polymxa</u>	50

(7.5-15% acrylamide, coomassie blue stain)

Plate 4.1.3. Native-PAGE (zymogram stain)

Sample		µg loaded
A)	40-60% $(NH_4)_2SO_4$ precipitate	60
B)	Concentrated crude enzyme	60

(7.5-15% acrylamide, coomassie blue stain)

fractions. This was later shown to be due to autolysis during electrophoresis as inactivation of the enzyme with 10 mM EDTA prior to boiling with SDS prevented autolysis and resulted in consistency in the number of bands observed (see Figure 4.1.8). The profile obtained before and after $(NH_4)_2SO_4$ fractionation (Plate 4.1.4) shows the absence of protein bands above the major band (enzyme band) indicating the removal of these proteins during this purification step.

4.1.2 Hydrophobic interaction chromatography

A phenyl Sepharose column (Section 2.15.3) was used to assess the use of hydrophobic interaction chromatography as a technique for purification. Dialysed samples of the 40-60% $(NH_4)_2SO_4$ precipitate were loaded onto the column. Hydrophobic interaction chromatography separates proteins on the basis of the differing strength of hydrophobic interactions with an uncharged bed material containing hydrophobic groups. Elution is obtained with a decreasing salt gradient, the most hydrophobic proteins eluting last. No enzyme activity was detected in the fractions eluted with a 4-2 M NaCl gradient in 50 mM Tris-HCl pH 8.0. The enzyme was eluted with a 2-0 M NaCl linear gradient (Figure 4.1.4). The peak fraction of protease activity eluted at approximately 1.3 M NaCl. Pooled fractions were dialysed against 50 mM Tris-HCl pH 8.0 and the total activity was measured. The remaining activity was about 33% of starting activity. Hydrophobic interaction was considered an unsatisfactory technique for purification because of the significant loss of activity.

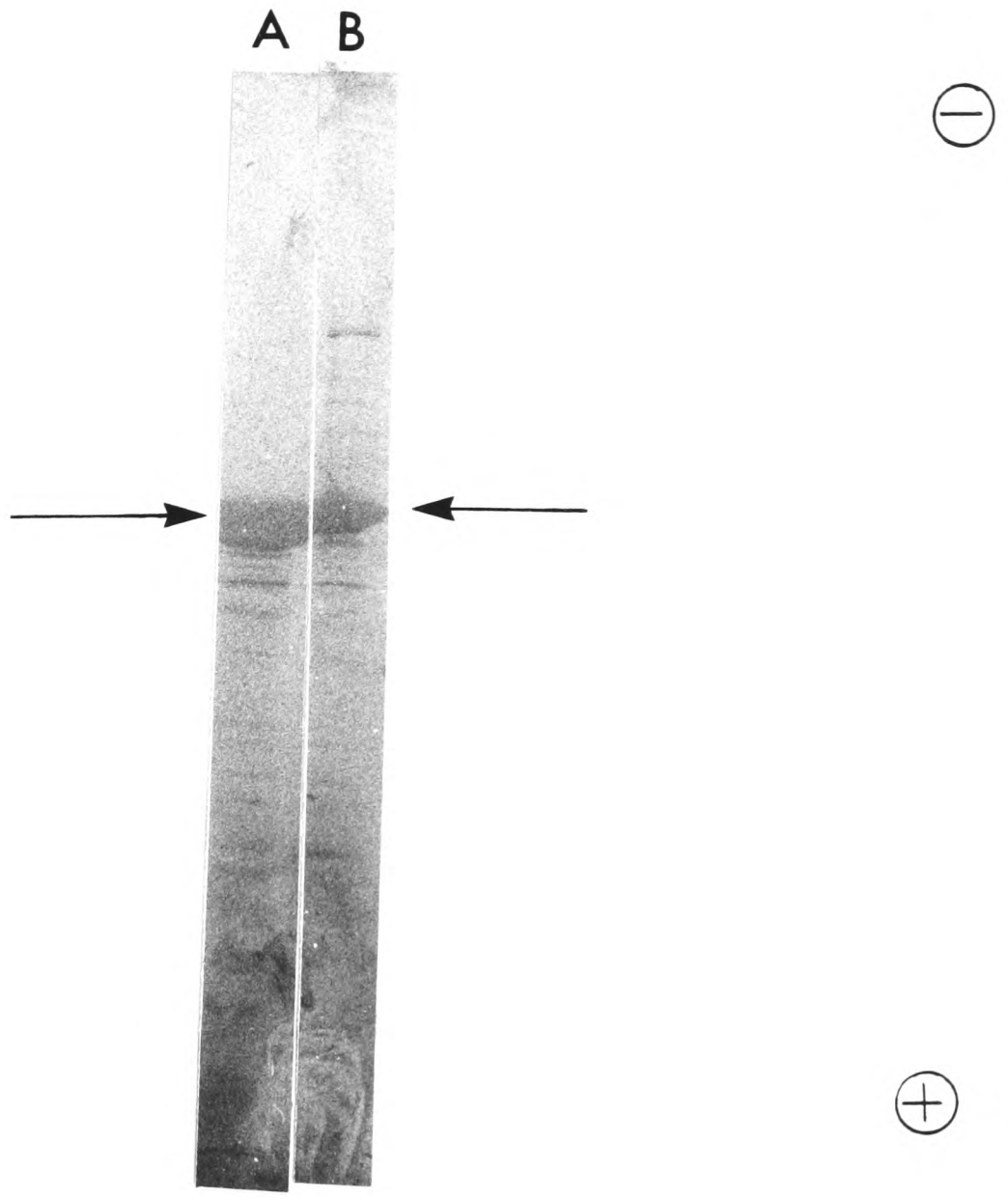


Plate 4.1.4. SDS-PAGE of the concentrated enzyme and 40-60% precipitate

		µg loaded
A)	40-60% pellet of $(NH_4)_2SO_4$	25
B)	Concentrated crude enzyme	25

(7.5-15% acrylamide, silver stain, EDTA treated enzyme)

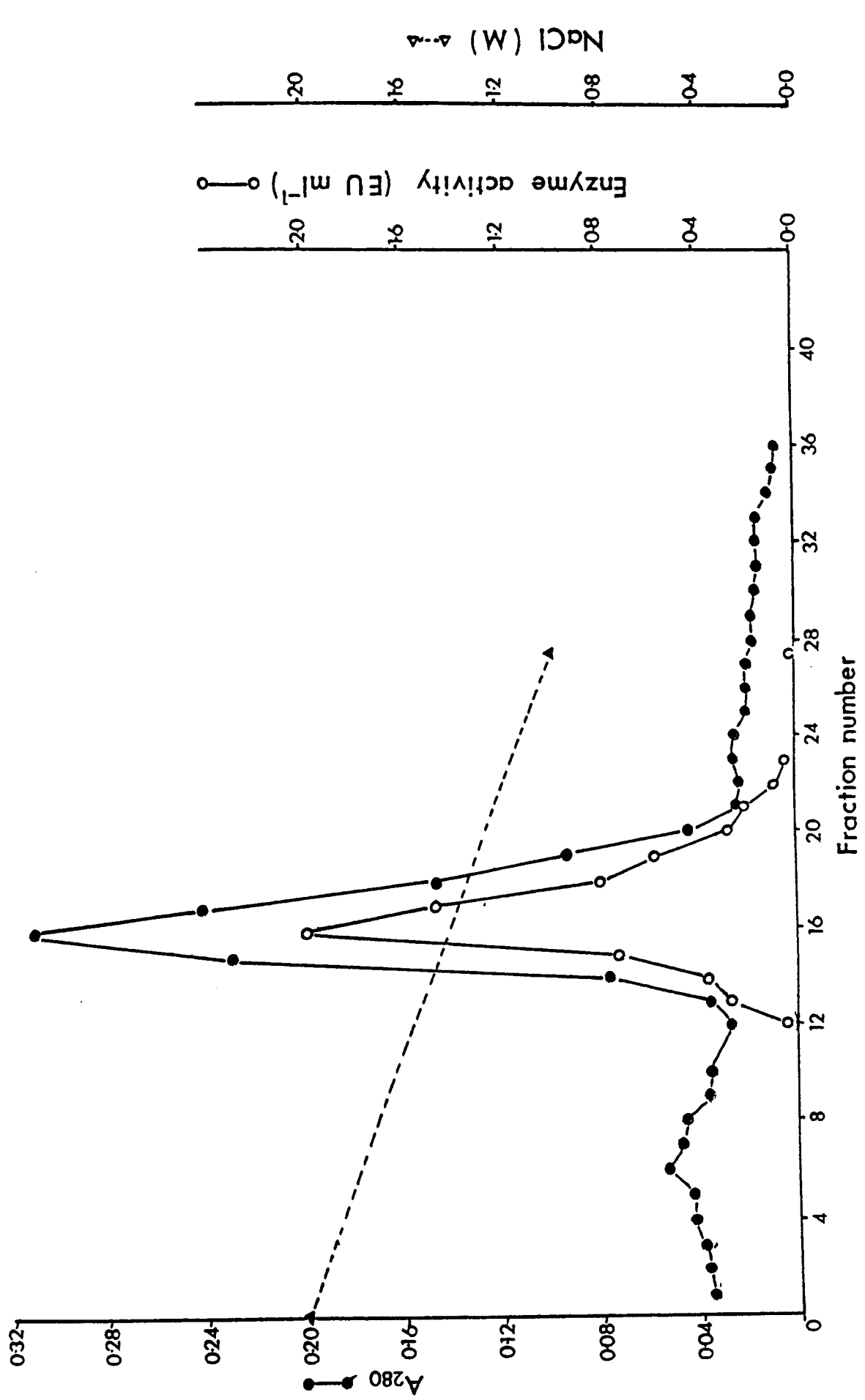


Figure 4.1.4. Phenyl column chromatogram of 40-60% $(NH_4)_2SO_4$ precipitate

Sample: 40-60% $(NH_4)_2SO_4$ precipitate (3.5 ml, 2.7 mg protein ml^{-1})
Buffer: 50 mM Tris HCl, pH 8.0
Gradient: 2.0-0.00 M NaCl
Fraction volume: 4.0 ml
Flow rate: 9.0 ml h^{-1}

4.1.3 Ion exchange chromatography

DEAE Sepharose CL 6B was chosen as a suitable matrix. It has the capacity for relatively high flow rates and good resolution. The buffer used was 50 mM Tris-HCl pH 8.0 because previous work (Patel et al., 1985; Fairbairn & Law, 1986) reported the isoelectric point (PI) of the extracellular proteases from Pseudomonas fluorescens to be around pH 6.0. Dialysed samples after $(NH_4)_2SO_4$ precipitation were loaded, washed and eluted with a linear gradient of 0-0.5 M NaCl within the buffer. Fractions were monitored for protein and enzyme activity.

The profile obtained after ion exchange chromatography of the 40-60% $(NH_4)_2SO_4$ precipitate is given in Figure 4.1.5.

Two peaks of activity were detected. Peak A contained approximately 60% of the total activity while peak B contained about 15% of the activity. Peak A was eluted at approximately 0.18 M NaCl while peak B eluted at about 0.23 M NaCl. A large peak of non-enzymic protein was eluted after the two protease peaks providing evidence for the separation of the enzyme from other contaminating proteins. The bulk of non-enzymic protein was eluted at 1 M NaCl in the ion exchange buffer. The fractions of the two peaks were pooled separately, concentrated against polyethylene glycol and dialysed against the ion exchange buffer.

Samples of both peaks were analysed by SDS-PAGE to monitor the progress of purification. One major protein band was seen in both peaks as well as several low molecular weight bands (Plate 4.1.5). However the EDTA treated

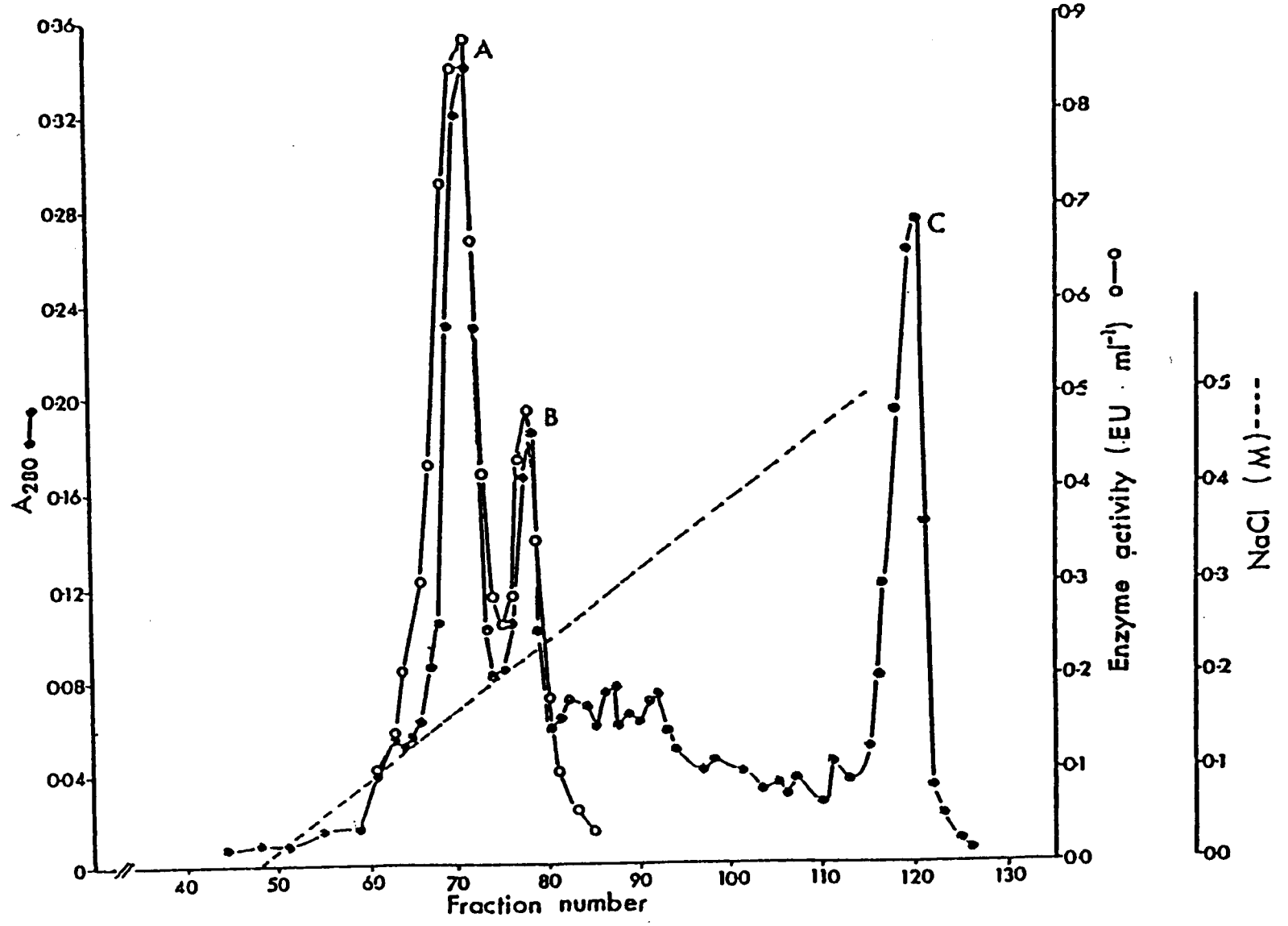


Figure 4.1.5. Ion exchange chromatography of 40-60% $(NH_4)_2SO_4$ precipitate

Sample: 40-60% $(NH_4)_2SO_4$ precipitate (5 ml, 4.8 mg protein ml^{-1})
Buffer: 50 mM Tris HCl, pH 8.0
Gradient: 0.0-0.5 M NaCl
Fraction volume: 6 ml
Flow rate: 10 ml^{-1}

A - peak A
B - peak B
C - non-enzymic protein

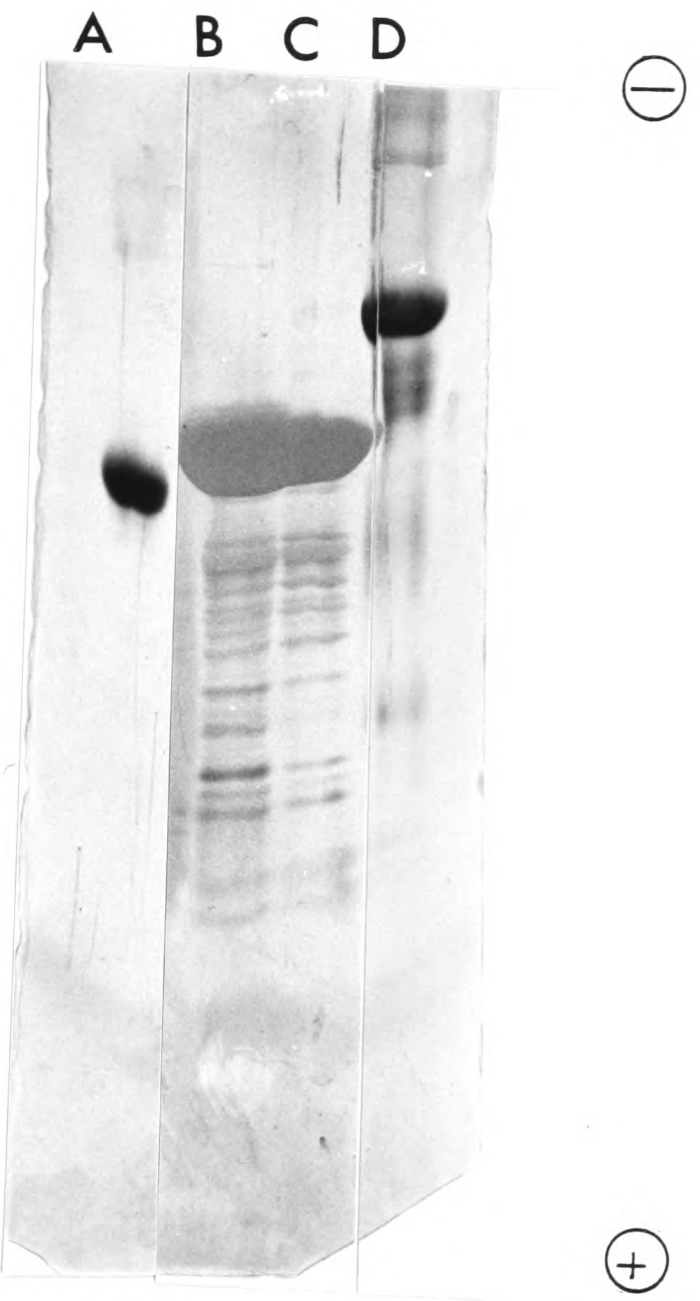


Plate 4.1.5. SDS-PAGE of the two peaks after ion exchange chromatography

		µg loaded
A)	Ovalbumin	2.5
B)	Peak B	25
C)	Peak A	25
D)	Bovine serum albumin (BSA)	2.5

(7.5-15% acrylamide, silver stain, active enzyme)

sample of peak A gave a single protein band when examined by SDS-PAGE (Plate 4.1.6). The disappearance of the low molecular weight bands proved that there was autolytic degradation during electrophoresis. The EDTA treated sample of peak B also gave a single protein band.

A zymogram stain was carried out to locate the enzyme (peak A) on a native gel (Plate 4.1.7). It was observed that the amount of substrate bound to the gel increased when the percentage of acrylamide decreased. Electrophoresis was carried out at 4°C to minimise autolysis. The enzyme did not migrate and remained near the top of the resolving gel. This confirms the problem of aggregation observed in Section 4.1.1. The problem of aggregation could not be resolved using a continuous gel. The enzyme remained near the top of the gel.

4.1.4 Gel filtration chromatography

Chromatography on Sephadex G-75 separates proteins on the basis of their molecular weight. Gel filtration of samples was carried out in Tris-HCl, 50 mM (pH 8.0) containing 0.1 M NaCl to increase the ionic strength and to prevent possible ionic interaction with the gel matrix.

To assess the use of gel filtration chromatography after $(NH_4)_2SO_4$ fractionation and to compare between gel filtration and ion exchange chromatography as the third stage of purification, dialysed samples of 40-60% $(NH_4)_2SO_4$ were loaded onto the Sephadex G-75 column. The profile obtained is shown in Figure 4.1.6. The results indicated that the yield and purification were lower with the use of Sephadex G-75 than with the use of DEAE Sepharose CL 6B.

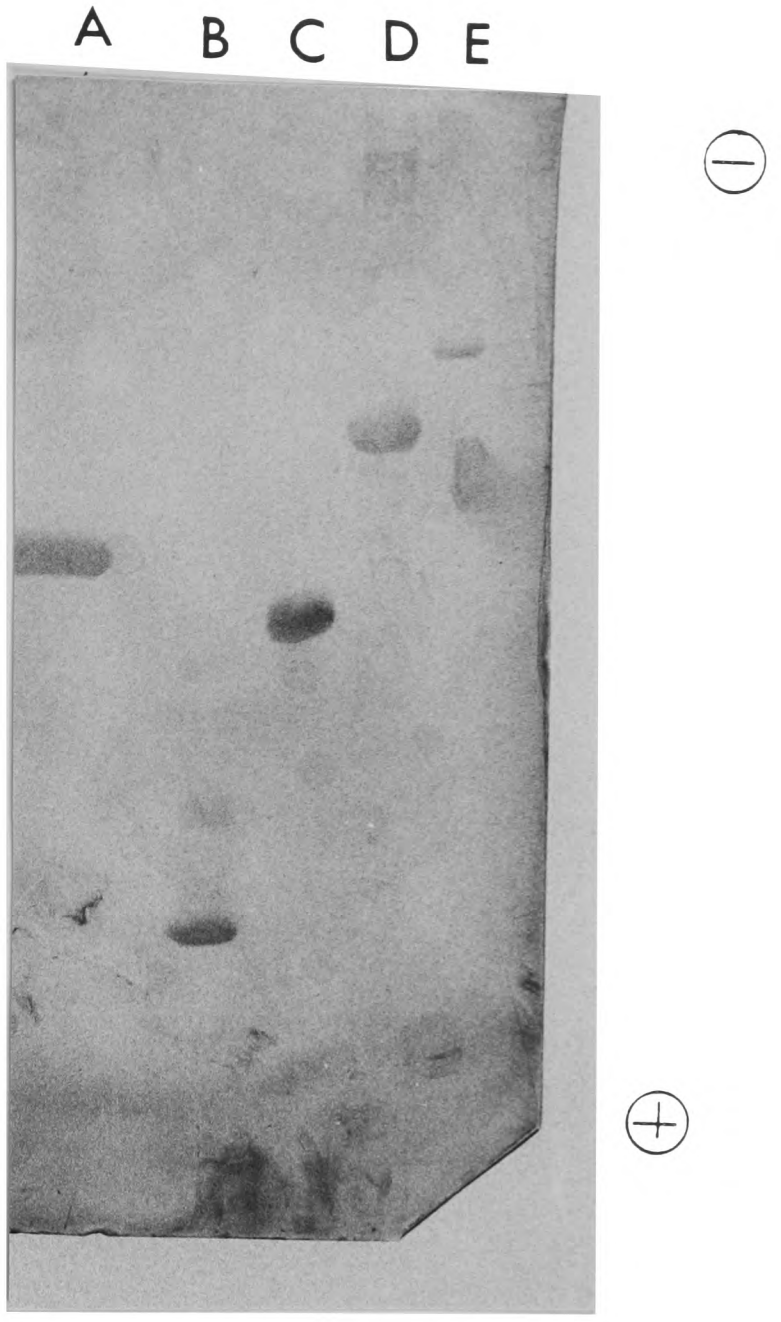


Plate 4.1.6. SDS-PAGE of Peak A

		µg loaded
A)	Peak A	25
B)	Cytochrome c	2.5
C)	Ovalbumin	2.5
D)	Bovine serum albumin	2.5
E)	Phosphorylase-b	2.5

(7.5-15% acrylamide, silver stain, EDTA treated samples)

Plate 4.1.7. Native-PAGE of Peak A after ion exchange chromatography

		Protein loaded (µg)
A)	Ovalbumin	50
B)	Peak A	50
C)	Zymogram stain of Peak A	50

(7.5-15% acrylamide, coomassie blue stain)

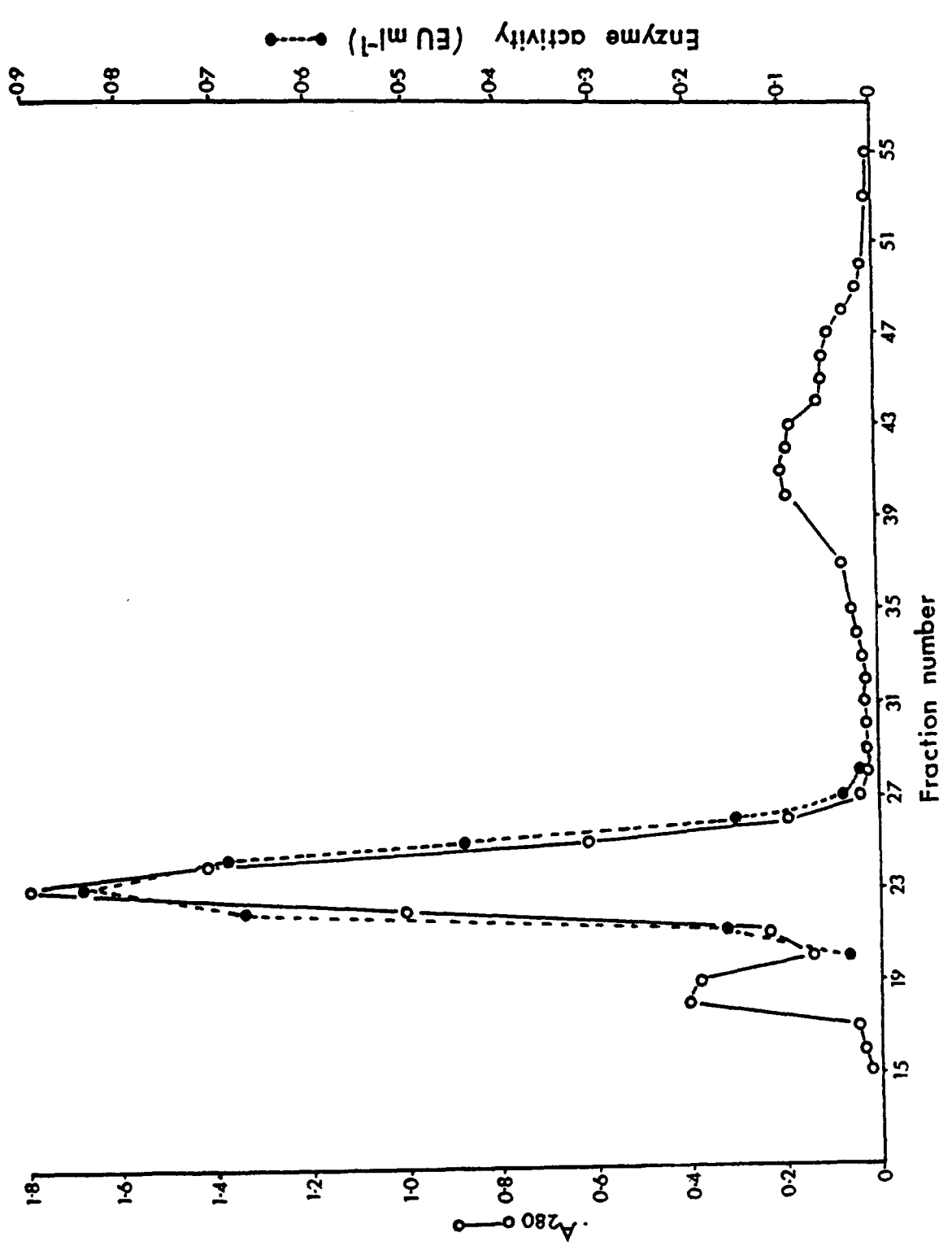


Figure 4.1.6. Gel filtration of 40-60% ($(NH_4)_2SO_4$ (3.0 ml, 5.6 mg protein ml^{-1} precipitated on Sephadex-G75

Sample: 40-60% precipitate
Buffer: 50 mM Tris HCl, pH 8.0 + 0.1 M NaCl
Fraction volume: 4.5 ml
Flow rate: 9.0 ml h^{-1}

4.1.5 Complete purification of the protease

A protocol for the purification of an extracellular protease from P. fluorescens R8 based on the results reported in the previous sections was selected. Ultrafiltration, ammonium sulphate fractionation and ion exchange chromatography were combined to produce a protocol for the purification. The enzyme peaks (A and B) were purified to homogeneity after ion exchange chromatography. However, gel filtration was used to assess the purity of peak A. A single peak of the enzyme protein was eluted (Figure 4.1.7), giving an indication that there was no contaminating protein in peak A. There was no increase in specific activity and it was considered that no further purification had been achieved. The sequence of purificiation is listed in Table 4.1.2. Samples from each stage of purification were analysed by SDS-PAGE to monitor the resolution of the protease from other contaminating proteins (Plate 4.1.8). In the present study, the enzyme has been purified in three stages which resulted in an approximately 3-fold increase in specific activity for both peaks and a yield of 38% (peak A) and 9.5% (peak B) of the original activity. The results obtained from this study indicate that the enzyme secreted by P. fluorescens R8 was a major component in the culture supernatant.

4.1.6 Gel permeation HPLC

Gel permeation HPLC was performed as described in Section 2.20.1. This technique was carried out to monitor the progress of purification.

The profile obtained in Figure 4.1.8 shows the

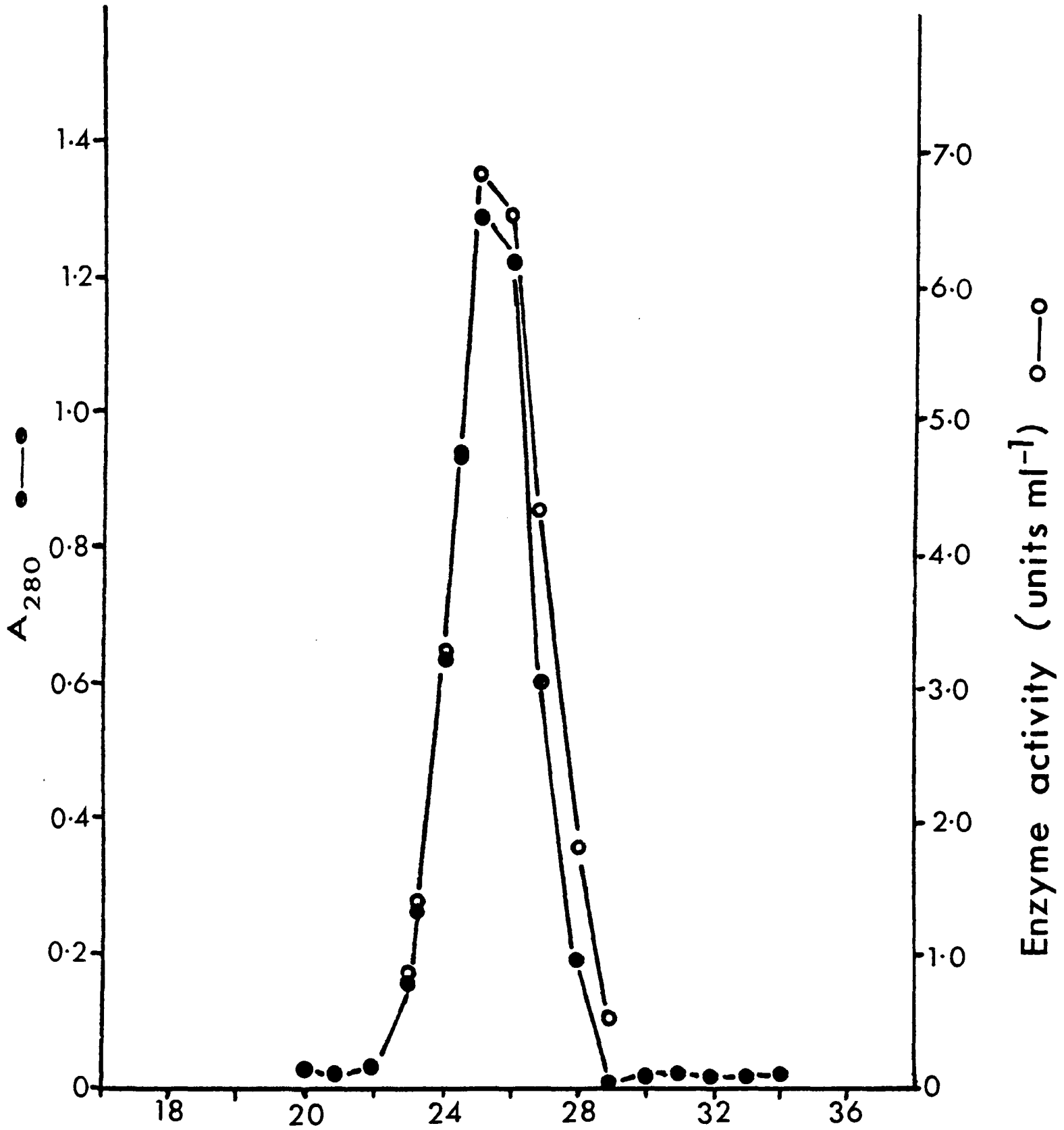


Figure 4.1.7. Gel filtration of peak A on Sephadex G-75

Sample: Peak A (4 ml, 1.27 mg ml^{-1})

Buffer: 50 mM Tris-HCl pH 8 + 0.1 M NaI

Fraction volume: 4.5 ml

Flow rate: 9.0 ml h^{-1}

Table 4.1.2 Complete scheme for purification of a protease from P. fluorescens R8

Purification stage	Volume (ml)	Protein (mg ml^{-1})	Specific activity (EU/mg protein	Total activity (U)	Total protein (mg)	Purification (fold)	Yield (%)
Culture supernatant	2500	0.27	4.93	3325	675	1.00	100
Millipore concentrate	520	0.92	5.8	2772	477	1.20	83.4
Ammonium sulphate (40-60%) precipitate	39	5.0	10.94	2133	195	2.22	64
Ion exchange							
Peak A	246	0.37	13.90	1262	91.02	2.82	38
Peak B	72	0.34	12.90	317	24.50	2.62	9.5

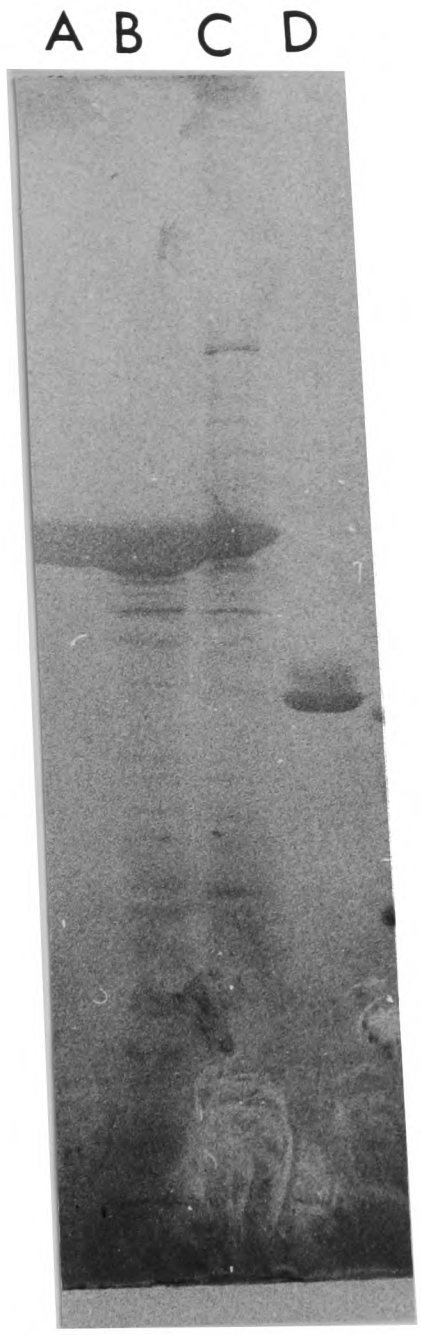


Plate 4.1.8. SDS-PAGE of samples from the purification procedure

		µg loaded
A)	Peak A after IE	27
B)	40-60% $(NH_4)_2SO_4$ precipitate	27
C)	Concentrated culture supernatant	27
D)	Carbonic anhydrase	2.5

(7.5-15% acrylamide, silver stain, EDTA treated samples)

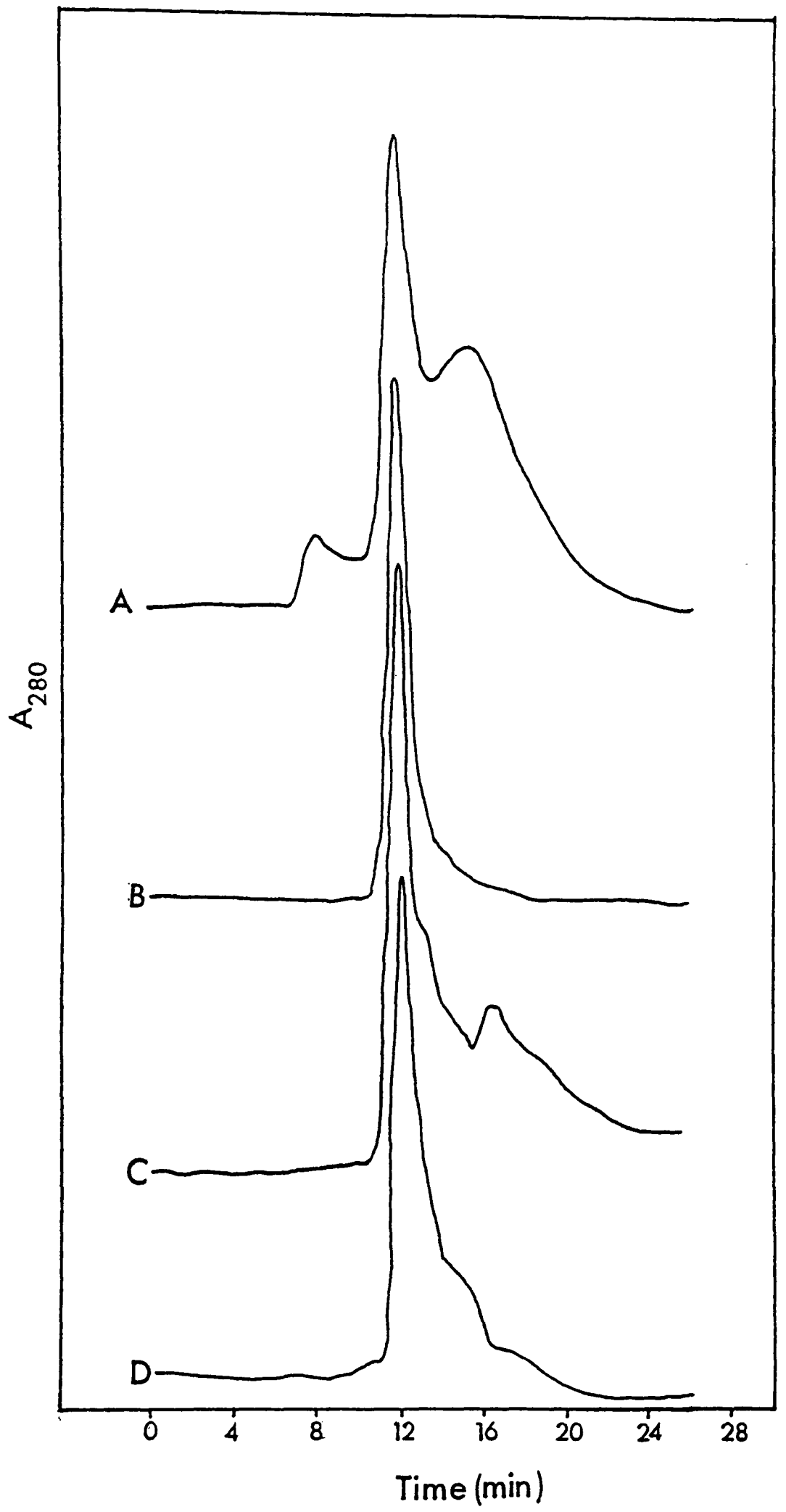


Figure 4.1.8. Gel permeation HPLC (GPHPLC) of different stages of purification

Buffer: 0.1 M phosphate pH 6.80
Flow rate: 0.8 ml/min
Pressure: 6-7 bar
Sample: ≈ 100 µg

A - 40-60% $(NH_4)_2SO_4$ precipitate
B - peak A after ion exchange
C - pooled fractions after phenyl Sepharose column
D - pooled fractions after Sephadex G-75

progress of purification. The two non-enzymic peaks (Figure 4.1.8, A) which eluted after approximately 8 and 17 min were not present after ion exchange chromatography. These results are in good agreement with the results obtained from SDS-PAGE (Plate 4.1.6) which indicated that there was a single protein band on the gel after ion exchange chromatography. Chromatogram C (Figure 4.1.8) shows that after hydrophobic interaction chromatography a non-enzymic peak was present which eluted after approximately 17 min. This indicates that the enzyme was not pure. The presence of a shoulder on the peak after Sephadex G-75 chromatography (Figure 4.1.8, D) also indicates that the enzyme was not pure. The results shown in Figure 4.1.8 indicate that ion exchange chromatography was more effective than either hydrophobic interaction or gel filtration chromatography as a single step procedure for purification of the protease. The results described in the previous sections (4.1.3 and 4.1.4) are in good agreement with the results obtained from gel permeation HPLC.

4.1.7 Discussion

The protease secreted by Pseudomonas fluorescens R8 has been purified in three steps: ultrafiltration, ammonium sulphate precipitation and ion exchange chromatography. Some proteases from Pseudomonas species have been purified to electrophoretic homogeneity in just two steps using ammonium sulphate precipitation followed by either gel filtration (Mayerhofer et al., 1973; Juan & Cazzula, 1976; Makino et al., 1981) or ion exchange chromatography (Noreau & Drapeau, 1979). Presumably the culture super-

natant used in these experiments contained few contaminating proteins other than the enzyme. Stepaniak & Fox (1985) also purified three extracellular proteases from Pseudomonas strain AF21 by using ion exchange and gel filtration chromatography. The enzyme protein studied here was eluted from the ion exchange column with approximately 0.2 M NaCl (0.18 peak A and 0.23 M NaCl peak B). Alichanidis & Andrews (1977) found that the protease produced by P. fluorescens NCDO 2085 was eluted from the DEAE-cellulose column with 0.20-0.25 M NaCl.

Two problems were encountered when gel electrophoresis was carried out to monitor the progress of purification and to locate the enzyme on the gel. These problems were autolysis and aggregation. The problem of autolysis could be resolved by inhibiting the enzyme prior to SDS-PAGE or by doing the native gel electrophoresis at 4°C. None of the attempts to resolve the problem of the enzyme aggregation on native gels were successful. Some other workers have reported problems during electrophoresis. Richardson (1981) found that electrophoresis carried out at room temperature (20-25°C) resulted in a decrease in the intensity of stained enzyme band and the appearance of several faster moving components which were due to autolytic degradation. Winters & Corpe (1971) and Amrute (1975) also had problems with the aggregation of an extracellular protease from Pseudomonas fluorescens W. They failed to elute the enzyme from DEAE-cellulose and other ion exchange media buffered at pH 8.0 in 0.01 M Tris-HCl. They interpreted this as being caused by the formation of high molecular

weight aggregates.

In retrospect, the enzyme was a major protein in the culture supernatant but because of autolysis, aggregation and lack of zymogram staining it was not possible to show this on native gels.

4.1.8 Summary

An extracellular protease from P. fluorescens R8 was purified to electrophoretic homogeneity in three stages. This protease was a major component in the spent growth medium. Two peaks were obtained after ion exchange chromatography. Peak A contained about 60% of the total activity while peak B contained about 15%. The purification scheme resulted in 3-fold purification in both peaks and a yield of 38 and 9.5% for peak A and peak B respectively.

4.2 Characterisation of purified protease (peak A)

The physicochemical and enzymic properties of the purified protease (peak A) were determined. The optimum conditions for enzyme activity, temperature, pH, the effect of inhibitors, heat stability, low temperature inactivation and substrate specificity were studied. In addition a number of other characteristics were also investigated.

4.2.1 Optimum temperature

The optimum temperature for enzyme activity was about 40°C when azocasein was used as a substrate (Figure 4.2.1). There was a rapid loss of activity at temperatures above 45°C. This is discussed in Section 4.2.12.

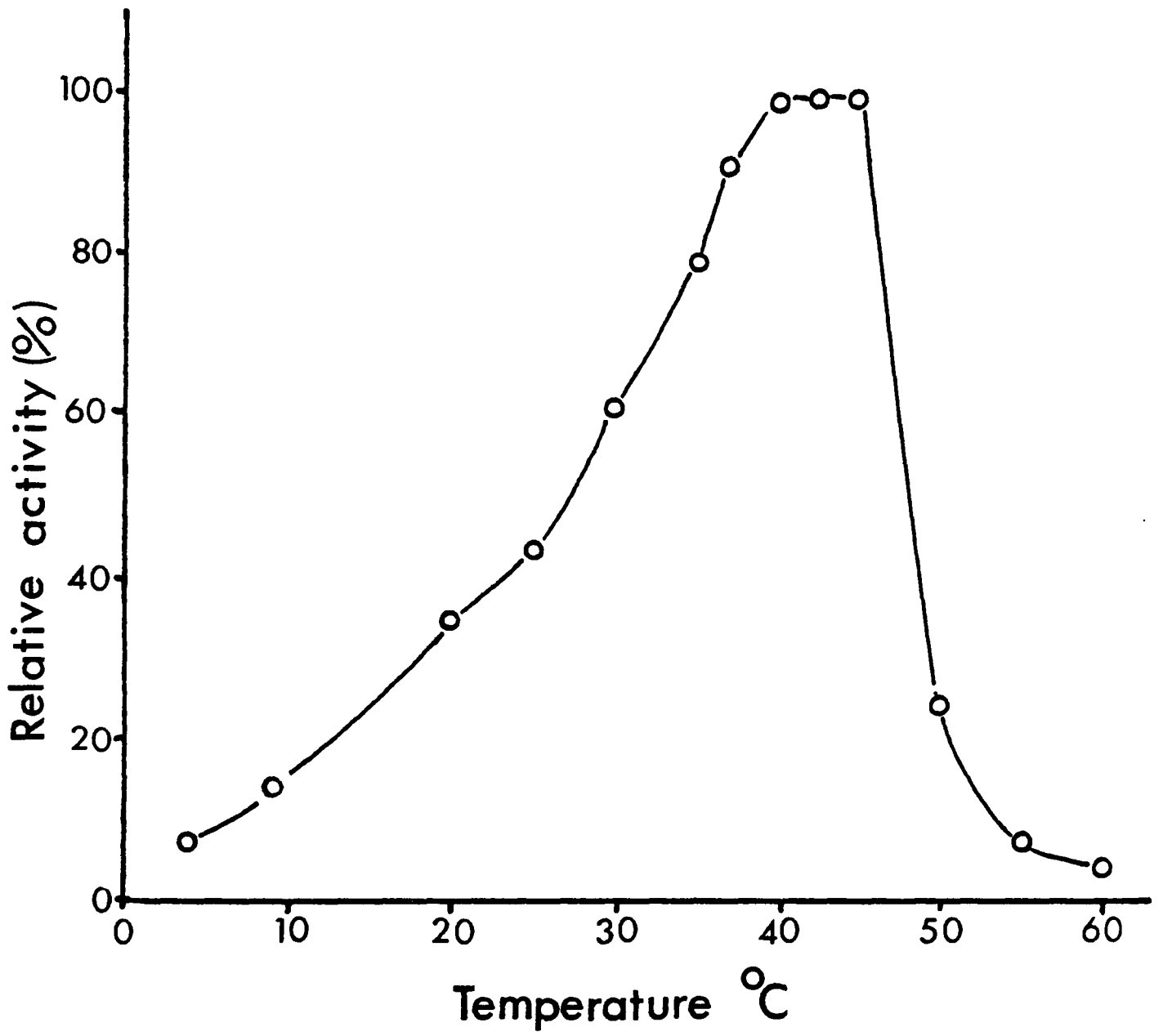


Figure 4.2.1. Effect of incubation temperature on enzyme activity with azocasein as substrate

4.2.2 Optimum pH

The optimum pH for the protease was found to be about pH 7.5 (Figure 4.2.2). The enzyme was active over a broad pH range, retaining > 80% activity between pH 5.5-8.5.

4.2.3 Effect of inhibitors

The following compounds were tested to ascertain their effect on protease activity: KCl, $MgCl_2$, $ZnCl_2$, $CaCl_2$, $CoCl_2$, $NiCl_2$, $FeCl_3$, $AlCl_3$, $MnCl_2$, phenylmethylsulphonyl fluoride (PMSF), iodoacetic acid, pepstatin A and NaCN. These were used at a final concentration of 1 mM. $AgNO_3$, $HgCl_2$, p-chloromercuribenzoate (PCMB) and N-tosyl-phenylalanylchloromethyl ketone (TPCK) were used at a concentration of 0.1 mM. Other reagents were tested at the following concentrations: urea (1-10 M), dithiotheriotal (DTT, 1 and 10 mM), trypsin inhibitor I-s (0.5 mg/ml), o-phenanthroline (1 and 10 mM), ethelenediaminetetracetic acid (EDTA, 1 and 10 mM), phosphoramidon (20 µg/ml) and ethylene glycol-bis (B-aminoethyl ether) N,N' tetra acetic acid (EGTA, 1 and 10 mM). Determination of the effect of metal ions and inhibitors was carried out as described in Section 2.28. The effect of metal ions on protease activity is shown in Table 4.2.1. Most of the metal ions had no effect on protease activity. However, incubation with $ZnCl_2$, $NiCl_2$ and $CoCl_2$ resulted in some loss of activity. EGTA (calcium-specific chelator), EDTA (metal chelator) and o-phenanthroline (zinc-specific chelator) were potent inhibitors of protease activity (Table 4.2.2.). The zinc chelator o-phenanthroline gave stronger inhibition of activity than either EDTA or EGTA; this indicates that the enzyme

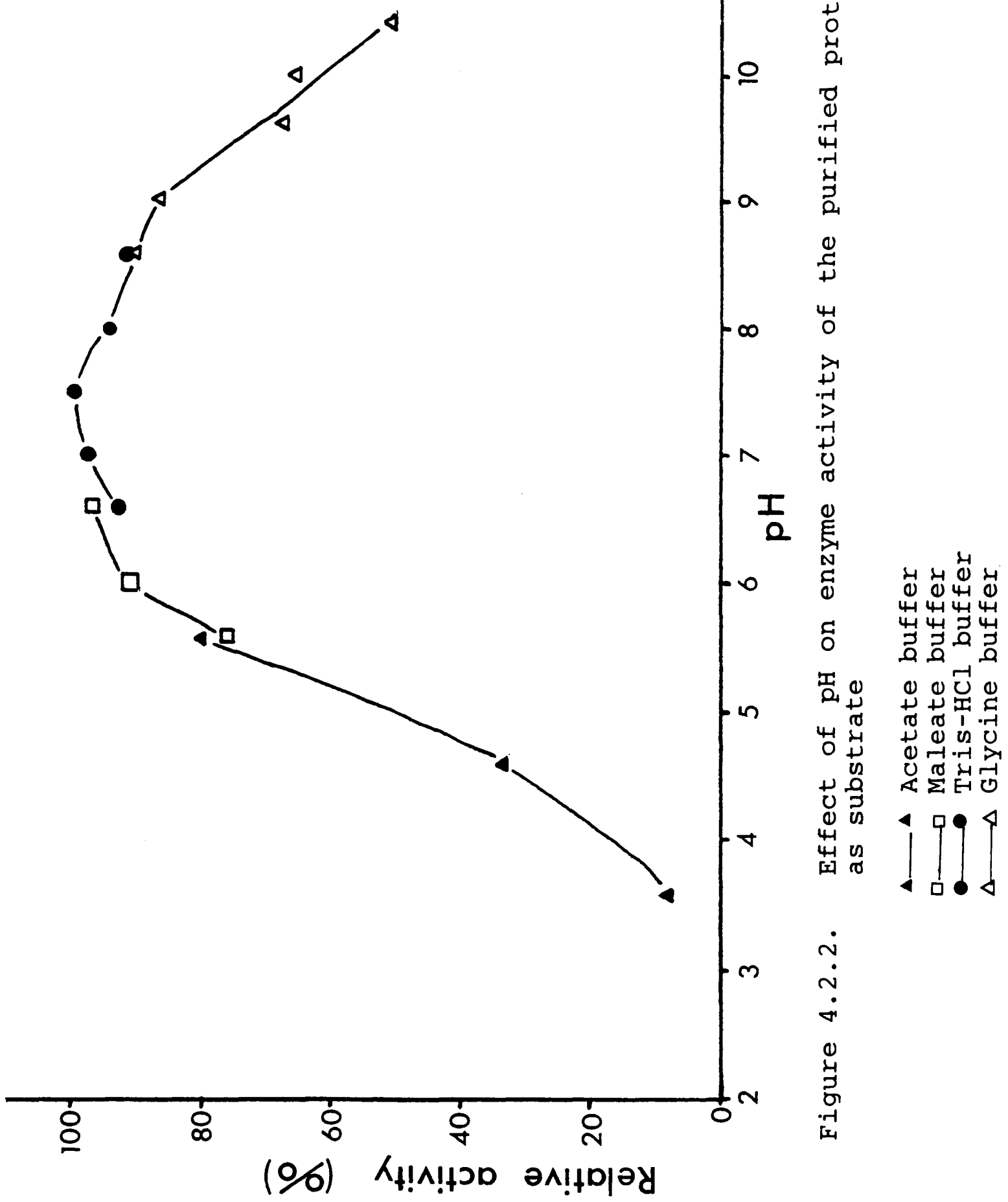


Figure 4.2.2. Effect of pH on enzyme activity of the purified protease with azocasein as substrate

Table 4.2.1 Effect of metal ions on the purified protease activity

Metal	Concentration (mM)	Residual activity (%)
$AgNO_3$	0.1	101
KCl	1	104
$MgCl_2$	1	97
$ZnCl_2$	1	57
$HgCl_2$	0.1	97
$CaCl_2$	1	100
$CoCl_2$	1	67
$NiCl_2$	1	57
$FeCl_3$	1	94
$AlCl_3$	1	100
$MnCl_2$	1	101

Table 4.2.2 Effect of inhibitor agents on the proteolytic activity of the purified protease

Inhibitor	Concentration (mM)	% control activity
L-cysteine	1	87
PMSF	1	100
TPCK	0.1	100
EGTA	1	65
EGTA	10	14
PCMB	0.1	100
Pepstatin A	1	100
Phosphoramidon	20 (µg/ml)	94
EDTA	1	46
EDTA	10	0.9
Iodoacetic acid	1	100
Sodium cyanide	1	100
O-phenanthroline	1	0.75
O-phenanthroline	10	< 0.01
Trypsin inhibitor	0.5 (mg ml^{-1})	91
Urea	1 (M)	100
Urea	4 (M)	32
Urea	10 (M)	< 0.01
DTT	1	83
DTT	10	38

contains zinc. PCMB which affects sulphydryl groups had no effect on the protease activity. PMSF, which acts as a pseudosubstrate and binds at the active site of serine protease, had also no effect on the activity. Reducing agents such as cysteine and DTT had only a slight effect. Urea (4 M) caused 68% inhibition whilst 1 M urea had no effect. Neutral protease inhibitors (phosphoramidon) and aspartic protease inhibitor (pepstatin A) did not inhibit the enzyme.

The influence of o-phenanthroline, EDTA, EGTA and DTT concentration on protease activity was also examined over a full range of reagent concentrations (Figure 4.2.3). o-phenanthroline was the most effective inhibitor. Only 4% of the activity remained when 0.5 mM o-phenanthroline was used.

4.2.4 Metal content

Determination of the metal content of the enzyme was carried out by using atomic absorbtion spectroscopy (Section 2.21). Results in Table 4.2.3 indicate that the enzyme contained approximately six atoms of calcium and two atoms of zinc per molecule and no copper or iron. The presence of these metals in the enzyme confirms the results obtained from studying the effect of different protease inhibitors (Section 4.2.3).

4.2.5 Carbohydrate content

Total neutral sugar was determined (Section 2.26). The total carbohydrate content was 0.12% and it was suggested that the enzyme was not a glycoprotein.

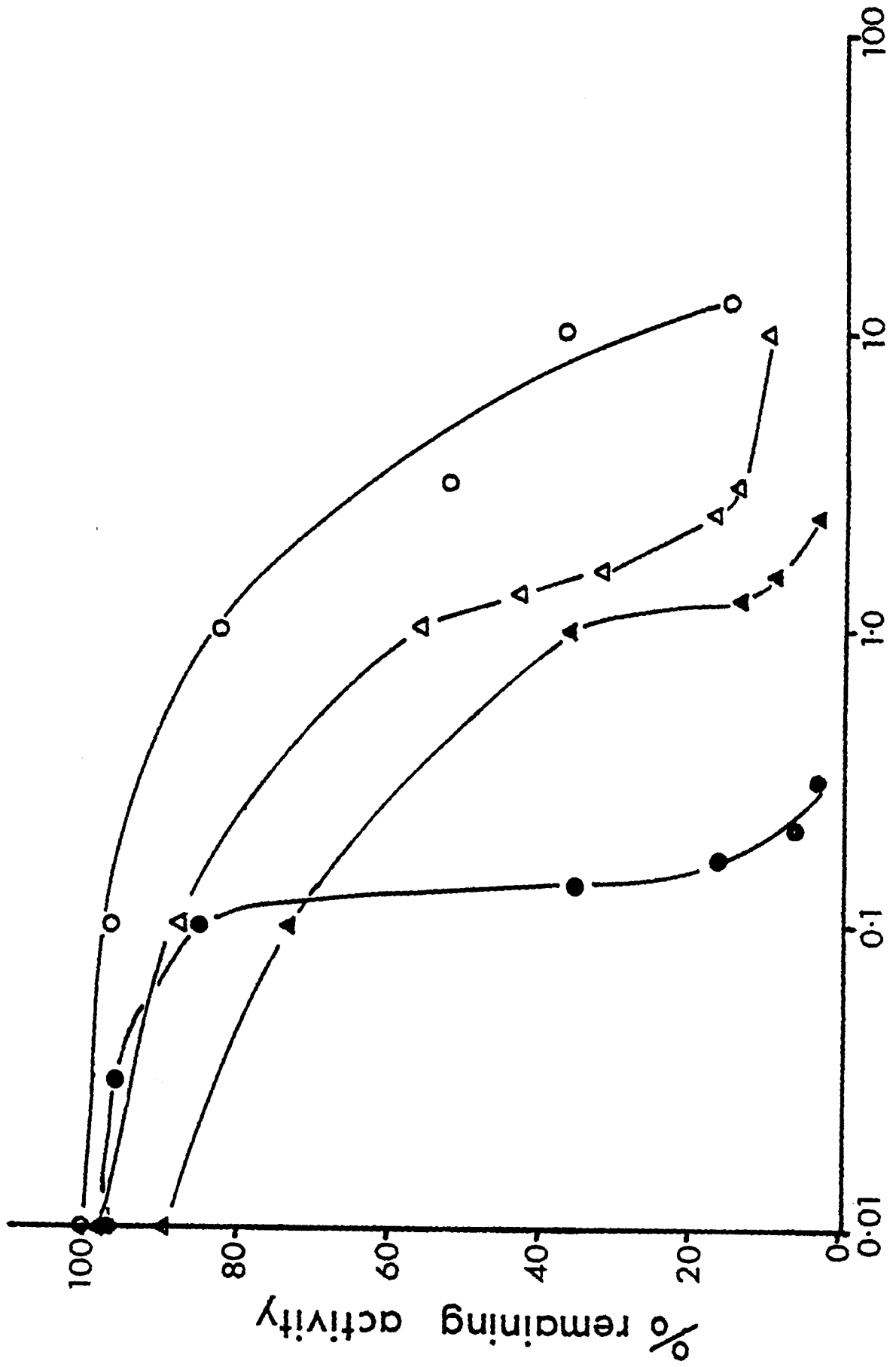


Figure 4.2.3. Effect of inhibitors on protease activity

Table 4.2.3 Metal content of the purified protease

Metal	Mg metal $g\ protein^{-1}$	Mole metal $mole\ protease^{-1}$
Ca	5.714	6.45
Zn	3.47	2.41
Cu	0.066	0.05
Fe	0.290	0.24

Assuming a molecular weight of 45 K daltons

4.2.6 Molecular weight

The molecular weight determination of the purified protease was carried out by three methods: gel filtration chromatography, SDS-PAGE and gel permeation HPLC. A calibrated column of Sephacryl S-300 gave a molecular weight of 45 K daltons (Figure 4.2.4). SDS-PAGE was performed as a second method to determine the molecular weight of the enzyme using different standards. The calibration curve (Figure 4.2.5) indicated that the enzyme had a molecular weight of 46 K daltons. When gel permeation HPLC was used, the relative retention time was consistent with the standard protein (ovalbumin), which has a molecular weight of 45 K daltons (Figure 4.2.6). There was therefore a good agreement between the three methods that the protease had a molecular weight of 45 ± 1 K daltons.

4.2.7 Molecular weight difference between peak A and peak B

An investigation was carried out to study the difference in molecular weight between the two enzyme peaks eluted after ion exchange chromatography, viz peak A and peak B. Samples of each peak were loaded onto the gel permeation HPLC column. The chromatogram obtained is shown in Figure 4.2.7. The profile obtained indicates that both peaks eluted at the same time (12 min), thus indicating peak A and peak B have identical molecular weights. This result is in good agreement with the findings obtained when SDS-PAGE was performed using different molecular weight proteins as standard. Both peaks gave the same relative

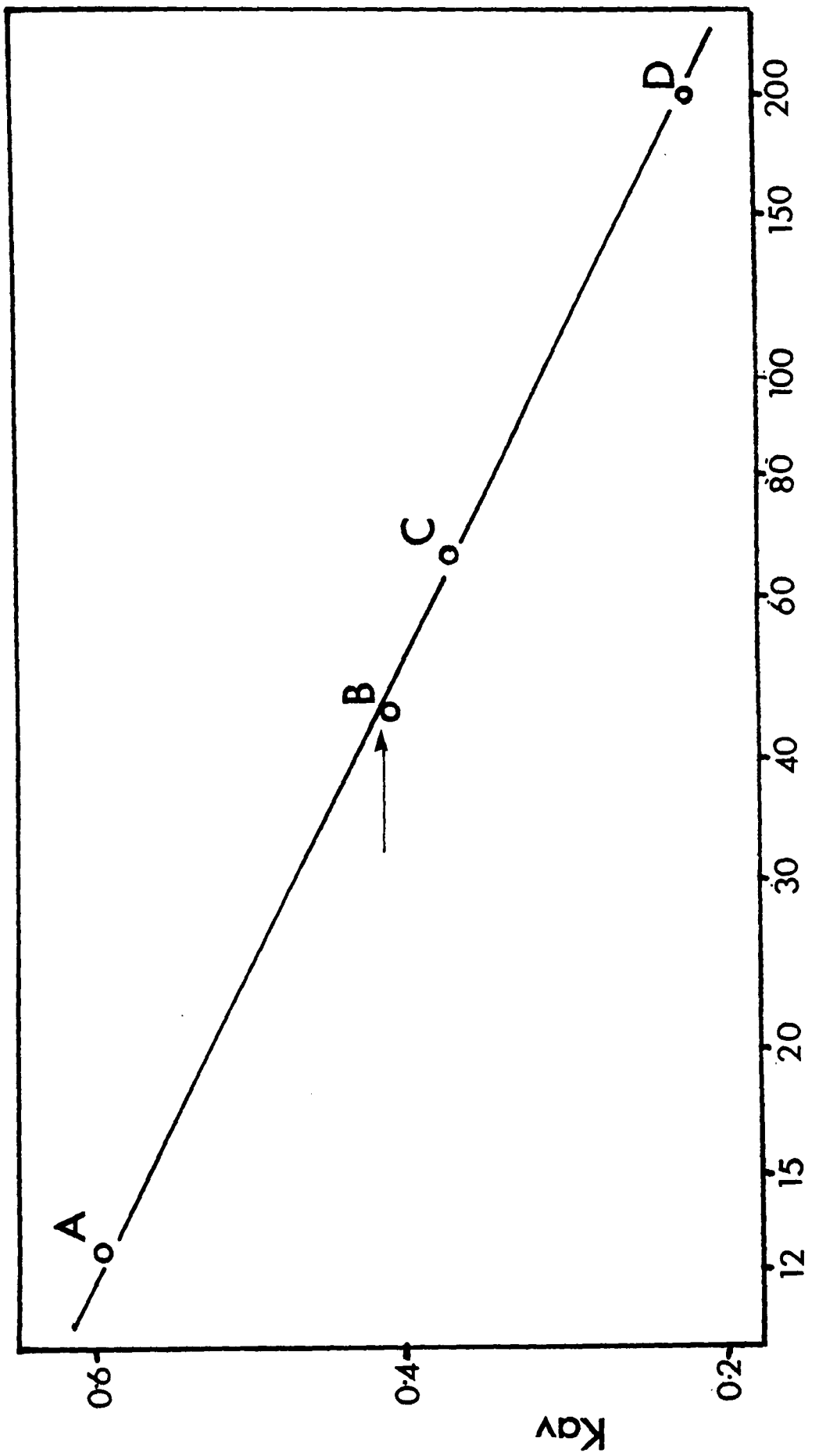


Figure 4.2.4. Molecular weight calibration curve for gel filtration on Sephacryl S-300

A - cytochrome c (12.4 K daltons)
B - ovalbumin (45 K daltons)
C - bovine serum albumin (66 K daltons)
D - β-amylase (200 K daltons)
↑ - peak A

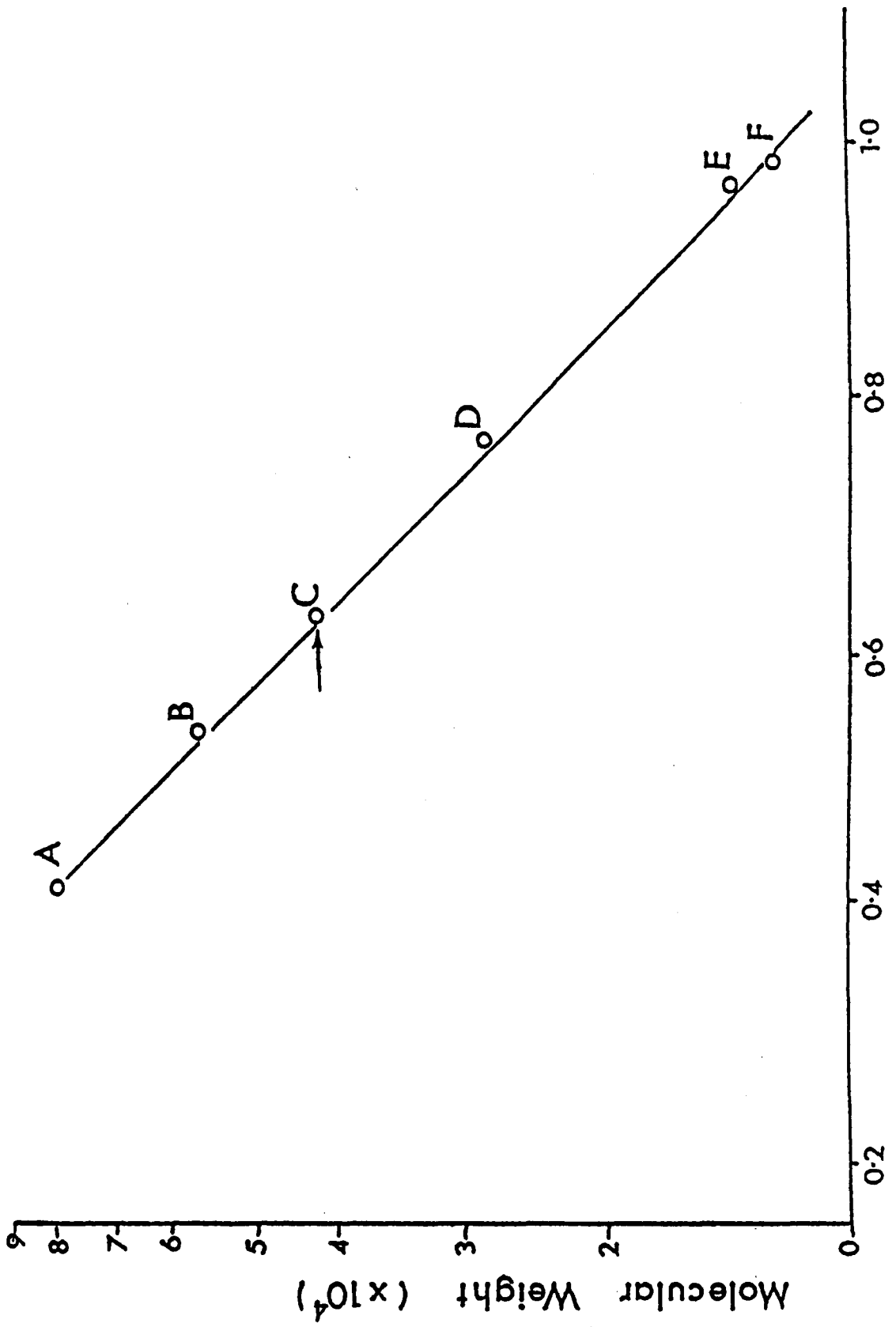


Figure 4.2.5. SDS-PAGE molecular weight calibration curve

A - phosphorylase-b (97.4 K daltons)
B - bovine serum albumin (66 K daltons)
C - ovalbumin (45 K daltons)
D - carbonic anhydrase (29 K daltons)
E - lysozyme (1.4 K daltons)
F - cytochrome c (12.4 K daltons)
↑ - peak A and peak B
(7.5-15% acrylamide)

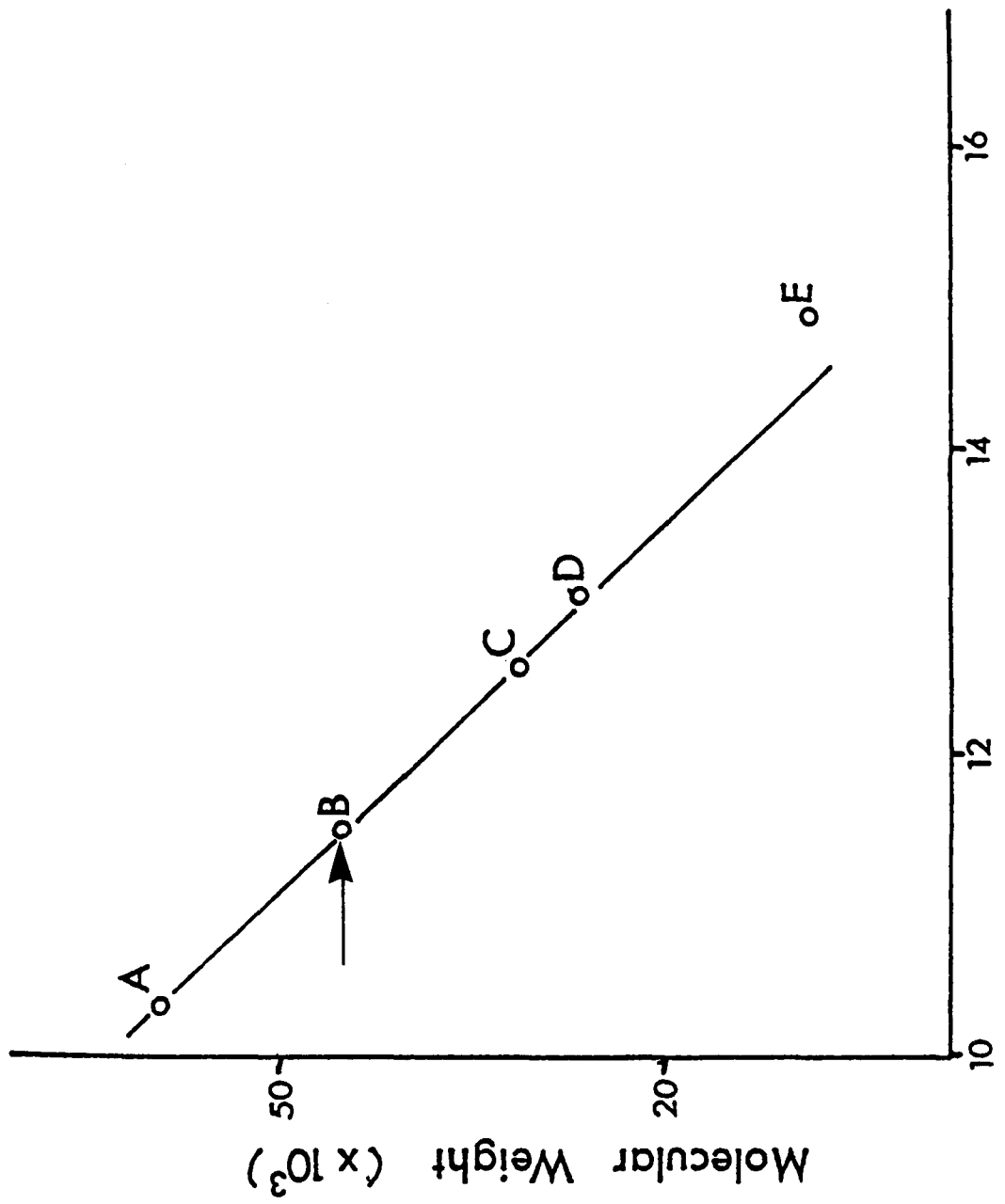


Figure 4.2.6. Molecular weight determination of the protease by HPLC gel permeation chromatography

A - bovine serum albumin (66 K daltons)
B - ovalbumin (45 K daltons)
C - carbonic anhydrase (29 K daltons)
D - chymotrypsinogen (24 K daltons)
E - lysozyme (14 K daltons)
↑ - peak A and peak B

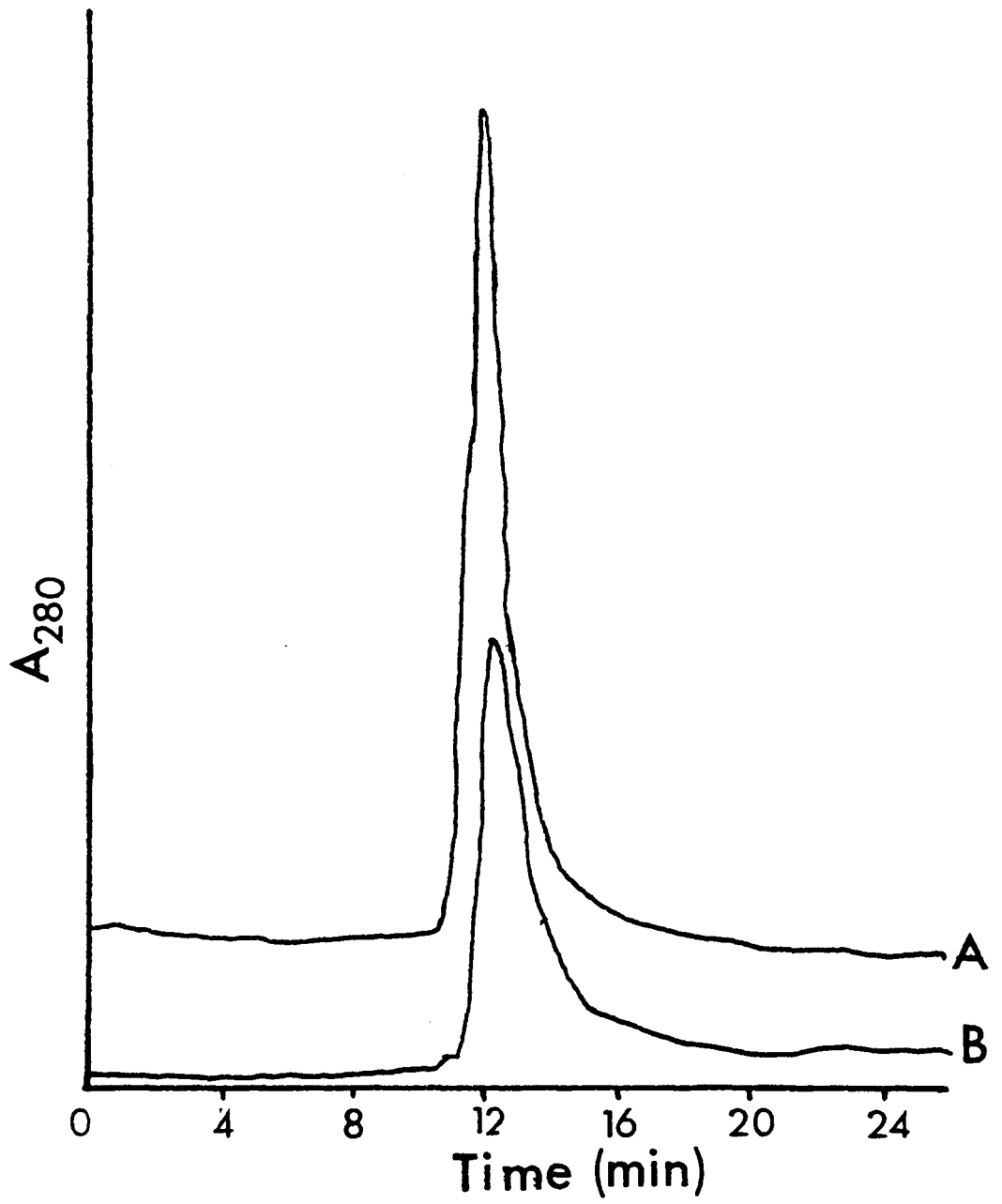


Figure 4.2.7. Gel permeation HPLC of:

A - peak A

B - peak B

mobility (Rf) see Figure 4.2.5, indicating that they have the same molecular weight of 45 ± 1 K daltons.

4.2.8 Tandem crossed immunoelectrophoresis (TCIE) for peak A and peak B

The immunochemical comparison between the two antigen samples peak A and peak B was carried out as described in Section 2.19.5. Although the precipitin peaks formed were not sharp and were detected in the section of the agarose which contained no antisera, the two peaks were found to be immunochemically identical.

4.2.9 Amino acid composition

Calculation of the amino acid residues after acid hydrolysis indicated that there were 469 amino acid residues per mol purified protease. The amino acid composition is given in Table 4.2.4. Aspartic acid and glycine were the most abundant amino acids. The enzyme did not contain cysteine and only four residues of methionine and tryptophan were in the enzyme molecule.

Average hydrophobicity (Bigelow, 1967) which is a measure of the hydrophobic nature of the protein was calculated from the overall amino acid composition. The figure obtained was 0.98 KCal/residue. Proteins with an average hydrophobicity of < 1.0 are generally considered to be hydrophilic.

4.2.10 N-terminal analysis

An attempt was made to determine the N-terminal amino acid of the protease by the dansylation technique (Section 2.24). This attempt did not succeed, probably because of the size of the enzyme (469 amino acid residues). However,

Table 4.2.4. Amino acid composition of the protease

Amino acid	Residues mol^{-1}
Lysine	16
Histidine	8
Arginine	7
Cysteic acid	0
Aspartic acid	66
Threonine	57
Serine	49
Glutamic acid	18
Proline	7
Glycine	64
Alanine	47
Half cystine	0
Valine	24
Methionine	4
Isoleucine	23
Leucine	31
Tyrosine	21
Phenylalanine	23
Tryptophan	4
Total number of residues	469

this method gives good results when low molecular weight proteins are used.

4.2.11 Substrate specificity

The substrate specificity of the protease was studied using a variety of proteins and synthetic substrates (Section 2.29). The results obtained are shown in Table 4.2.5. Whole casein or its fractions showed higher rates of hydrolysis than bovine serum albumin and cytochrome c; β-casein was found to be the most susceptible casein fraction. The enzyme did not hydrolyse β-lactoglobulin under the conditions described in Section 2.29. However, with some modifications β-lactoglobulin was attacked by the enzyme. The modifications were preincubation of β-lactoglobulin in 20 mM Tris-HCl pH 8.0 + 10 mM $CaCl_2$ for 30 min, increase in enzyme concentration (enzyme protein ratio 1:300) and increase of the reaction time from 30 min to 60 min. The breakdown of β-lactoglobulin by the protease was studied using RP-HPLC. This technique was chosen in preference to PAGE because of its speed and sensitivity. A major disadvantage of PAGE is that it is almost impossible to achieve separation of small peptides. RP-HPLC can rapidly resolve a mixture of peptides on the basis of hydrophobicity irrespective of size. By using this technique it was possible to analyse samples immediately after digestion therefore minimising the time of analysis and the possibility of further changes in the sample due to autolysis or proteolysis.

The profile obtained after using RP-HPLC to study the effect of the protease on β-lactoglobulin is shown in

Table 4.2.5. Hydrolysis of various proteins by the protease

Substrate	Relative activity (%)
Whole casein	100
β-Casein	62
α_{s1}-Casein	38
α_{s2}-Casein	38
Bovine serum albumin	5
Cytochrome c	14

The protease activity obtained in the presence of whole casein as a substrate was expressed as 100%. Activity detected in the presence of other substrates is relative to that obtained with whole casein.

Figure 4.2.8. More than 20 peaks were seen. In order to make sure that none of these peaks were due to autolysis of the enzyme a control was carried out. A sample of the enzyme alone was treated in the same way as in the assay conditions and loaded onto the RP-HPLC. One peak was obtained (Figure 4.2.9) indicating that no autolysis occurred under the assay conditions and that all the peaks when the enzyme was incubated with β-lactoglobulin were due to degradation by the enzyme.

The enzyme was unable to hydrolyse the following substrates: P-toluenesulphonyl-L-arginine methyl ester (TAME, trypsin substrate); N-acetyl-L-tyrosine ethyl ester (ATEE, chymotrypsin substrate) and N-t-Boc-L-leucine-P-nitrophenyl ester (estrase substrate).

4.2.12 Heat resistance

The effect of heat treatment on protease activity was tested by incubating the enzyme in 20 mM Tris-HCl at 140°C for various times (Figure 4.2.10). The enzyme activity decreased rapidly on initial heating and then levelled out producing a biphasic heat-inactivation curve. The D value (the time used to inactivate 90% of the enzyme) at 140°C was 1 min. When the enzyme was heated at 140°C for 5 sec (UHT treatment), 49% of the activity remained and 28% of the activity remained after heating the enzyme at 74°C for 17 sec (HTST treatment).

A sample of the enzyme was heated at 100°C for 5 min and then loaded onto the gel permeation HPLC column. The profile is shown in Figure 4.2.11. The boiled enzyme eluted after approximately 16 min while the unheated enzyme

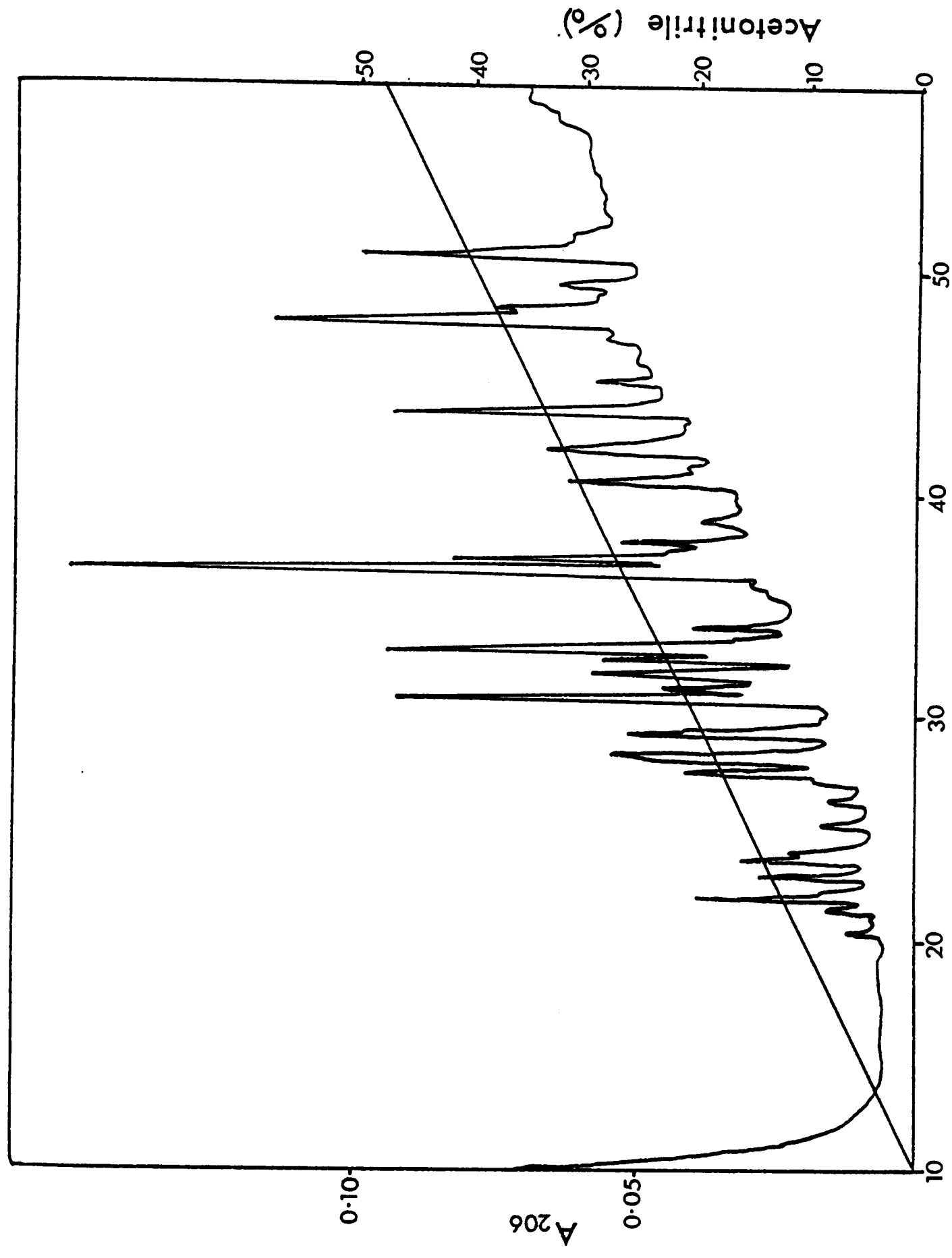


Figure 4.2.8. RP-HPLC chromatogram of β-lactoglobulin hydrolysed by the enzyme at pH 8·0

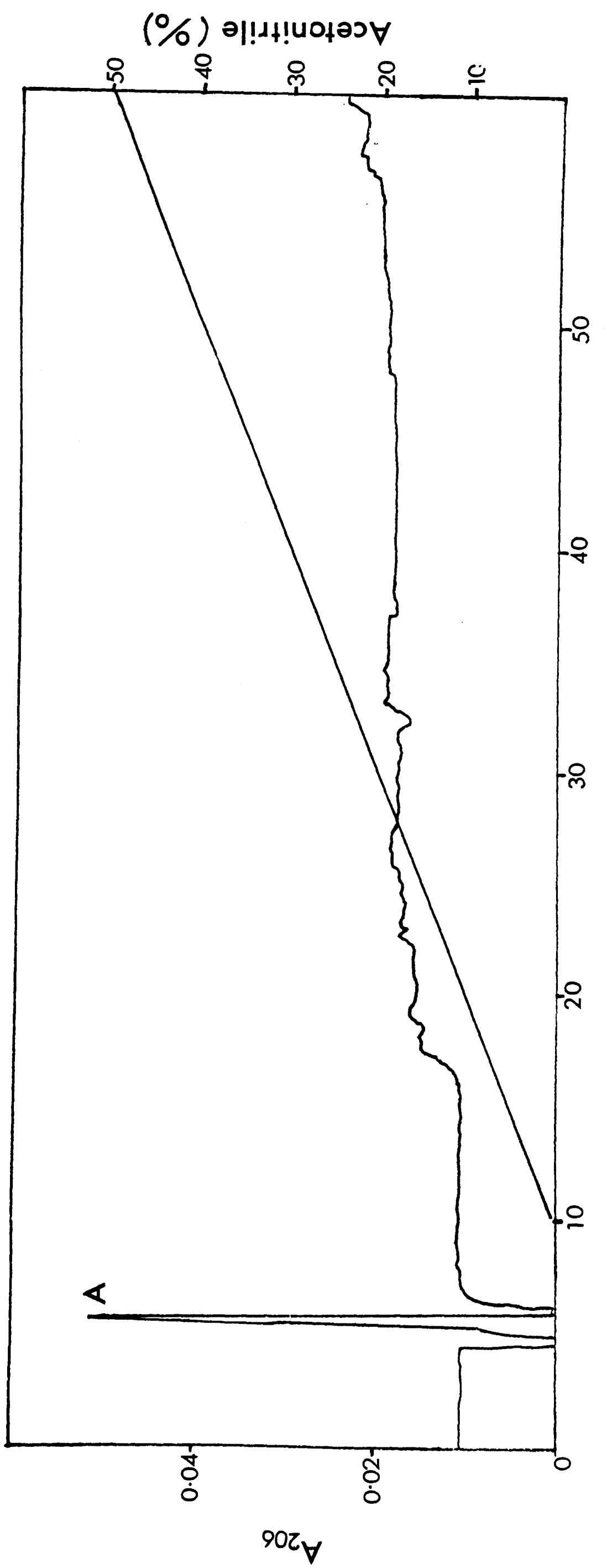


Figure 4.2.9. RP-HPLC chromatogram of the enzyme (40 µg) incubated at 37°C for 60 min in 50 mM Tris-HCl pH 8.0

A - enzyme/solvent peak

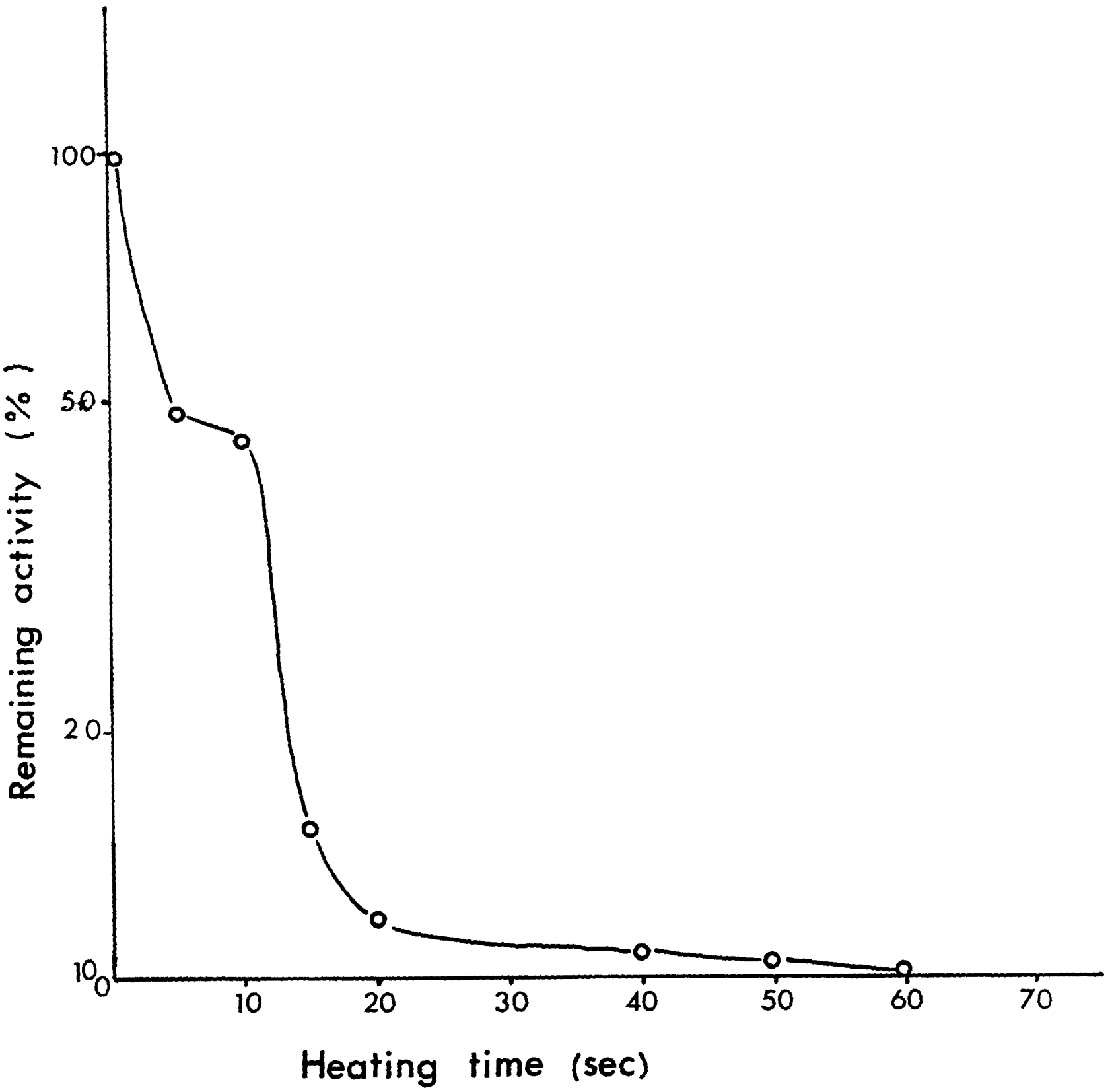


Figure 4.2.10. The heat inactivation of purified protease in 20 mM Tris-HCl buffer pH 7.5 at a temperature of 140°C

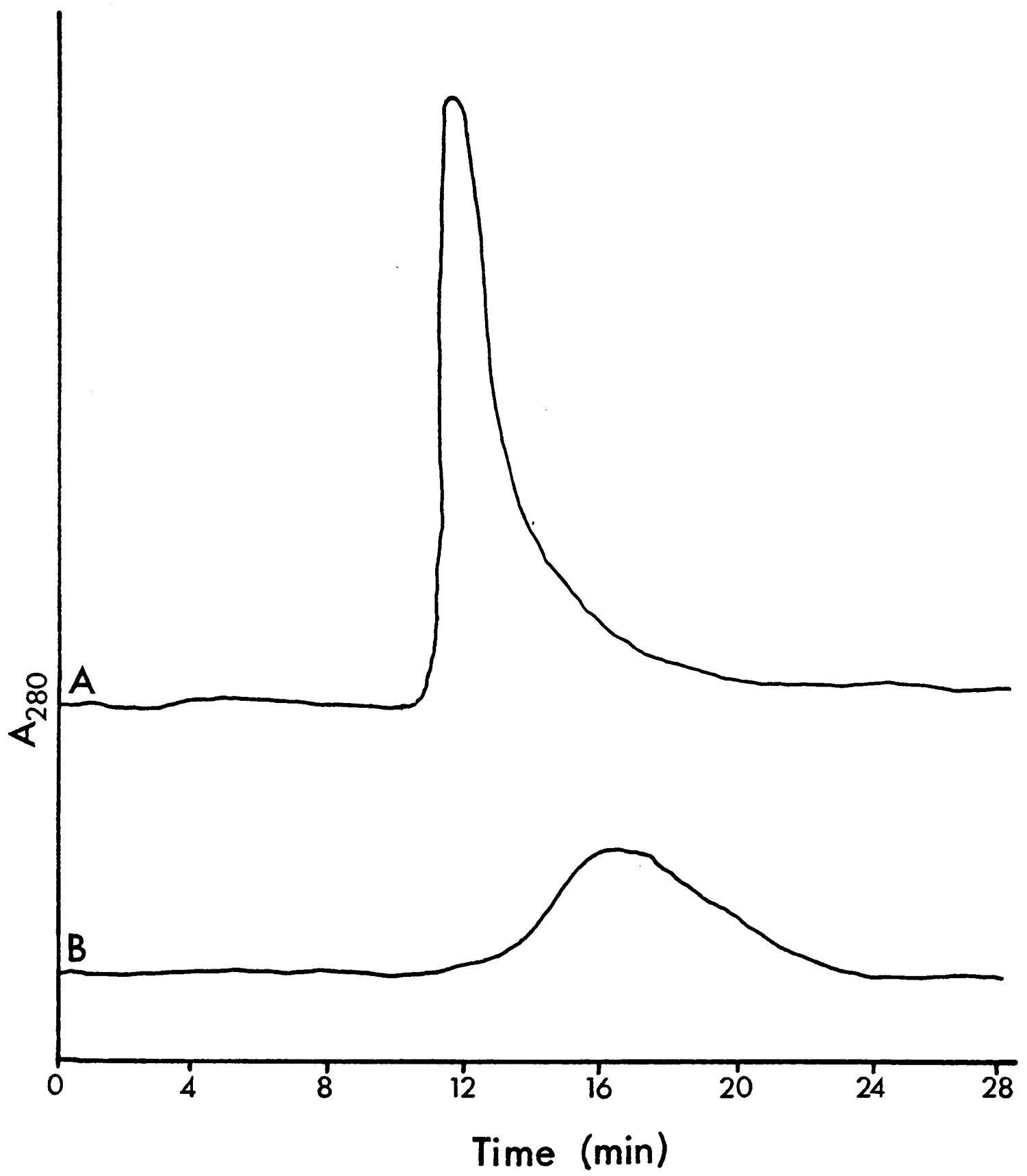


Figure 4.2.11. Gel permeation HPLC chromatogram of the enzyme (40 µg)

A - untreated enzyme

B - enzyme boiled for 5 min

eluted after 12 min. This indicates that the enzyme decreased in size probably because of autolysis. Another sample of the boiled enzyme was loaded onto the RP-HPLC. The profile obtained is shown in Figure 4.2.12. A large number of small peaks were seen indicating that the enzyme underwent autolysis when boiled for 5 min.

4.2.13 Low temperature inactivation (LTI)

The effect of heating the enzyme at 55°C for different periods of time was studied by using reversed-phase high performance liquid chromatography (RP-HPLC). It was used as a rapid technique to investigate the autolysis at 55°C. This method was very sensitive and small fragments could be detected. The enzyme was heated at 55°C for 5 min in 50 mM Tris-HCl pH 8.0; there was a rapid loss of enzyme activity and only 4% of the activity remained. The chromatogram obtained using RP-HPLC is shown in Figure 4.2.13. More than 25 peaks were observed and these were due to autolysis. There were no significant changes in the number of peaks seen when the enzyme was heated for 5, 30 and 60 min. The remaining activity was also the same. No difference in the number of peaks was observed between samples incubated at 55°C in the presence or absence of $CaCl_2$ (Figures 4.2.14 and 4.2.15). A control experiment was carried out to confirm that inhibition of the enzyme by preincubation with EDTA prevented autolysis. One peak only was observed, thus confirming that no autolysis occurred (Figure 4.2.16).

An investigation was carried out to study whether preincubation of the enzyme in the presence of $CaCl_2$ protected the enzyme against LTI. Samples of the enzyme

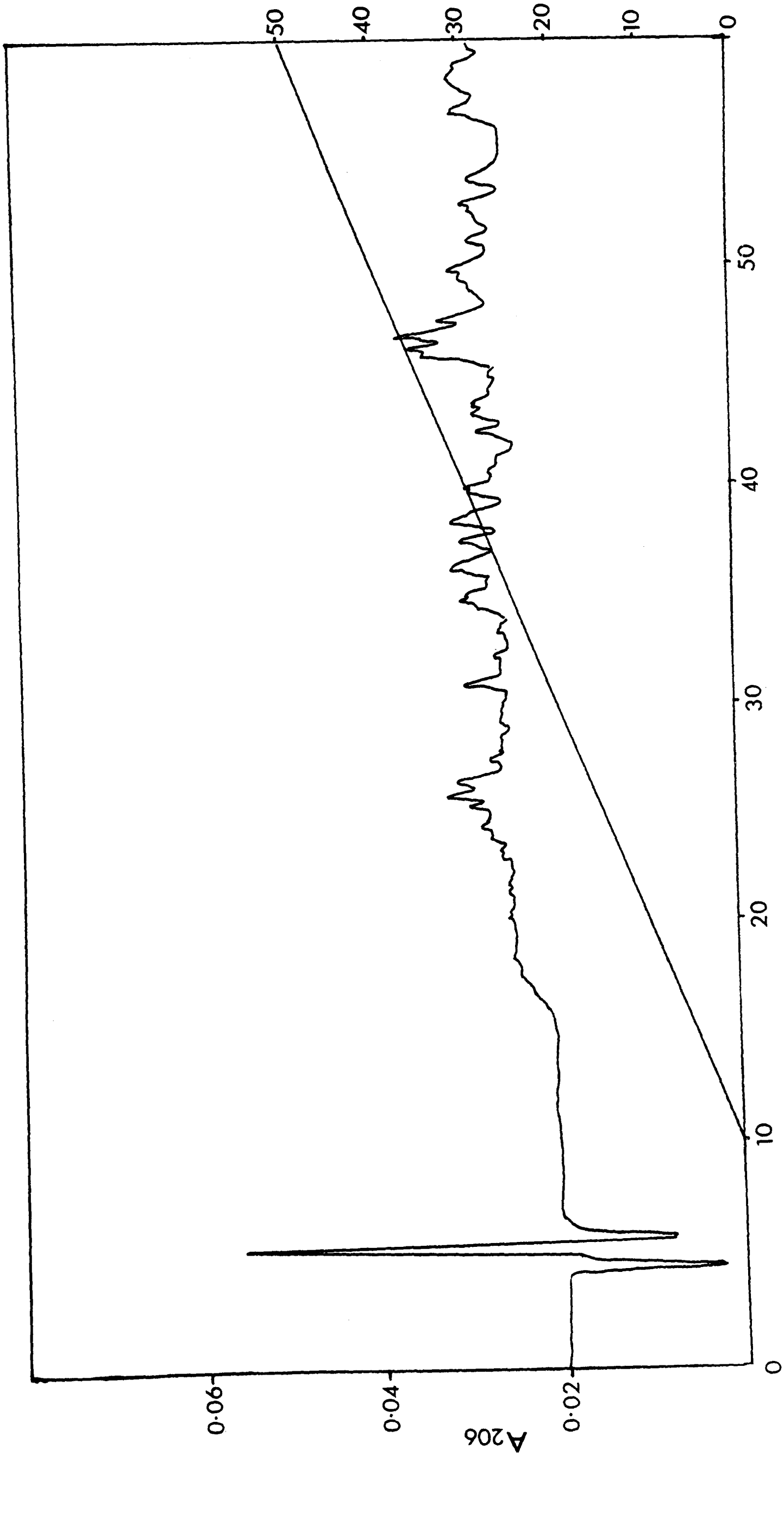


Figure 4.2.12. RP-HPLC chromatogram of boiled enzyme (40 µg) for 5 min

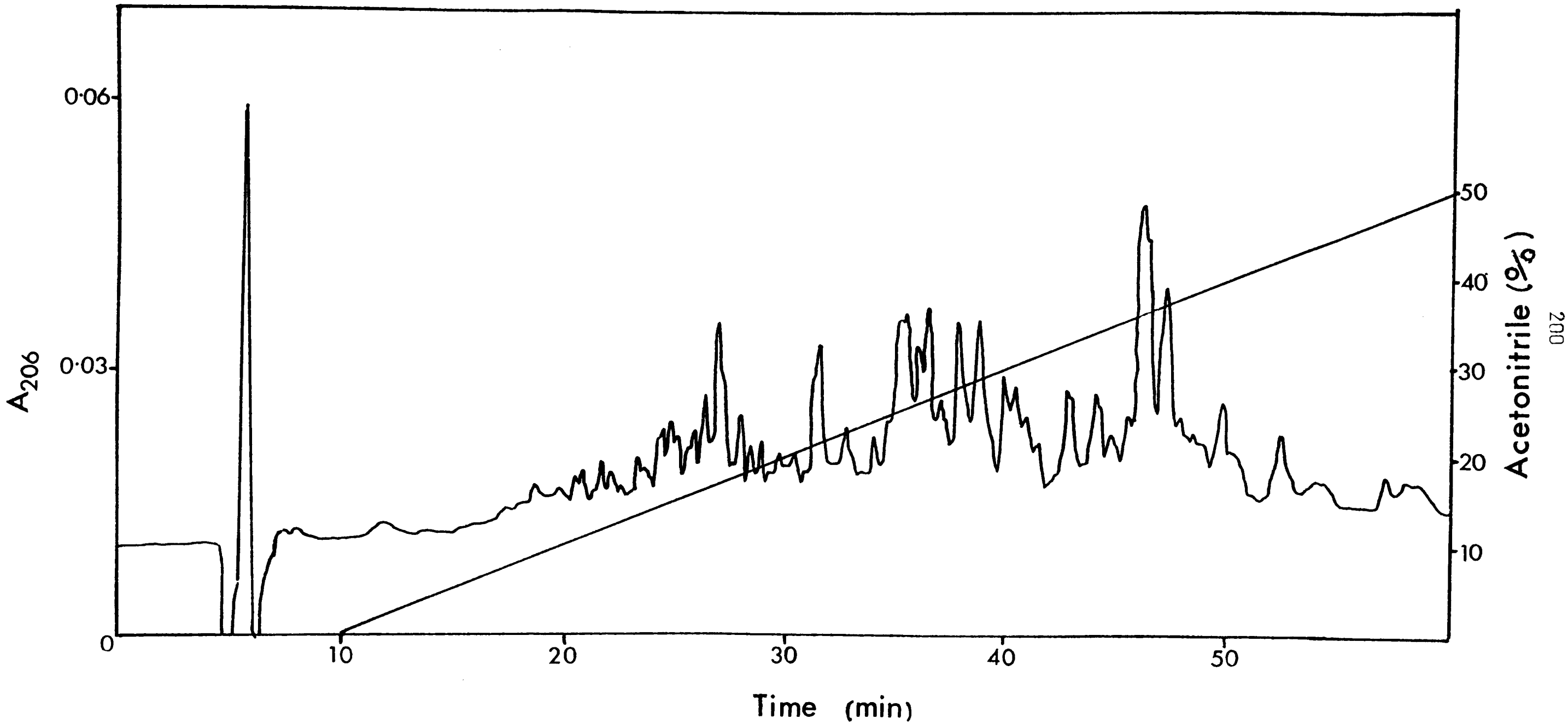


Figure 4.2.13. RP-HPLC chromatogram of enzyme (40 µg) incubated at 55°C for 5 min

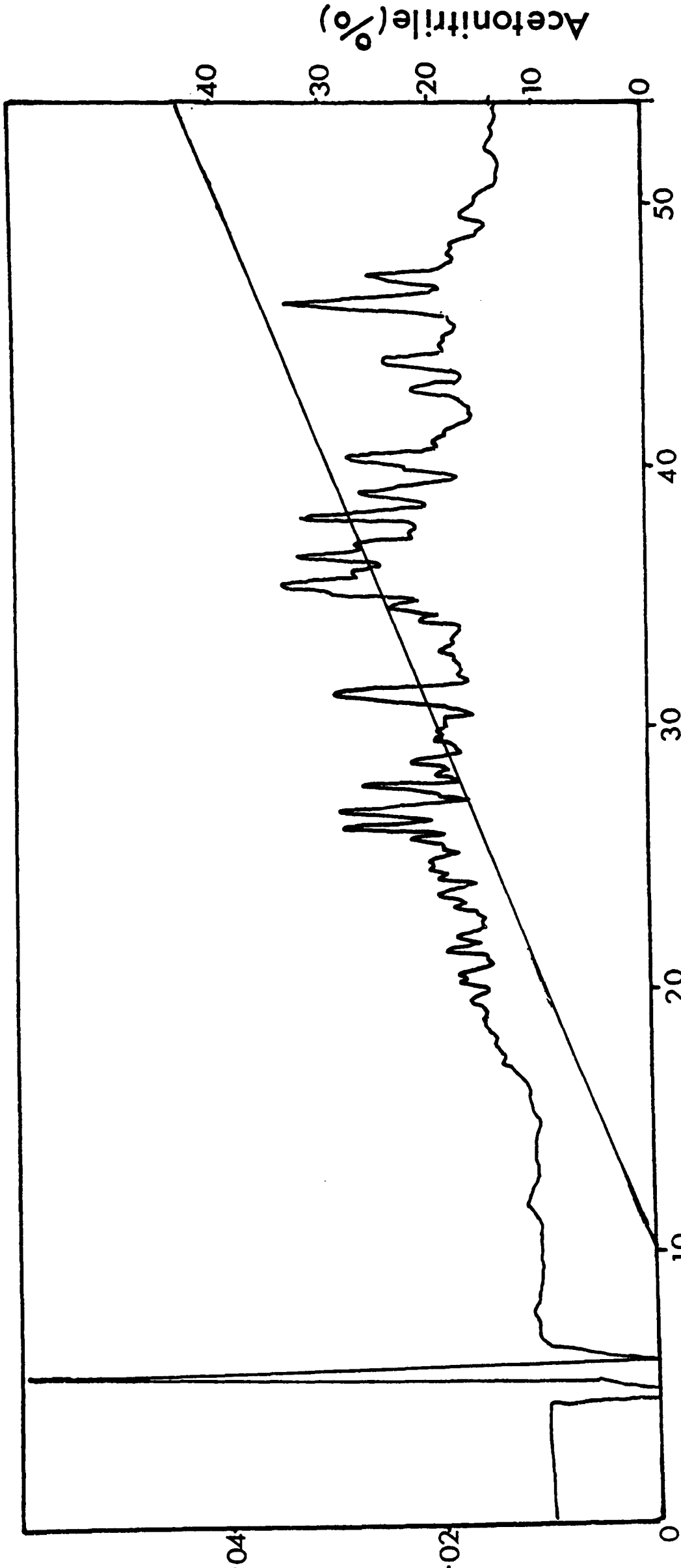


Figure 4.2.14. RP-HPLC chromatogram of enzyme (40 µg) incubated at 55°C for 30 min in the presence of $CaCl_2$

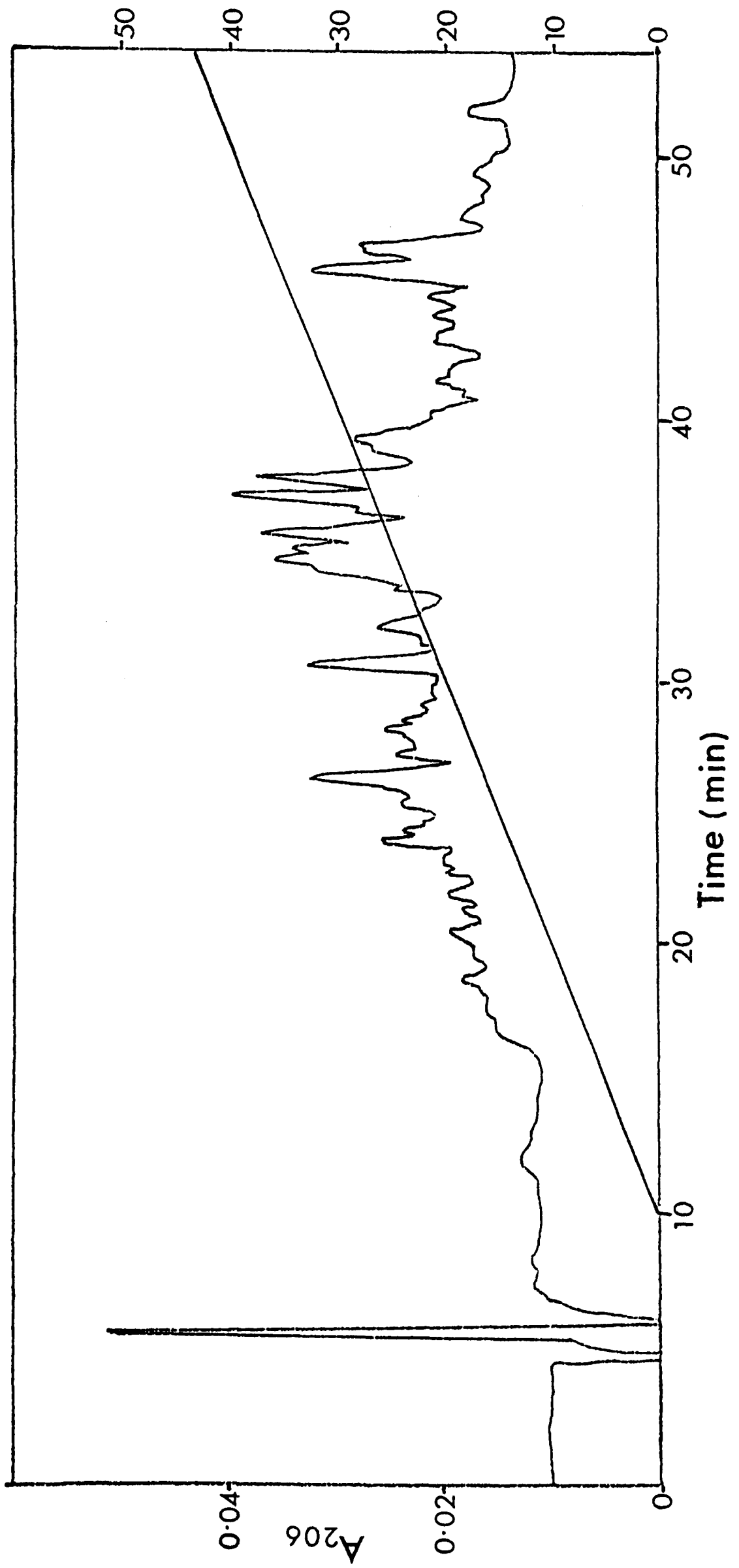


Figure 4.2.15. RP-HPLC chromatogram of the enzyme (40 µg) incubated at 55°C for 30 min in the absence of $CaCl_2$

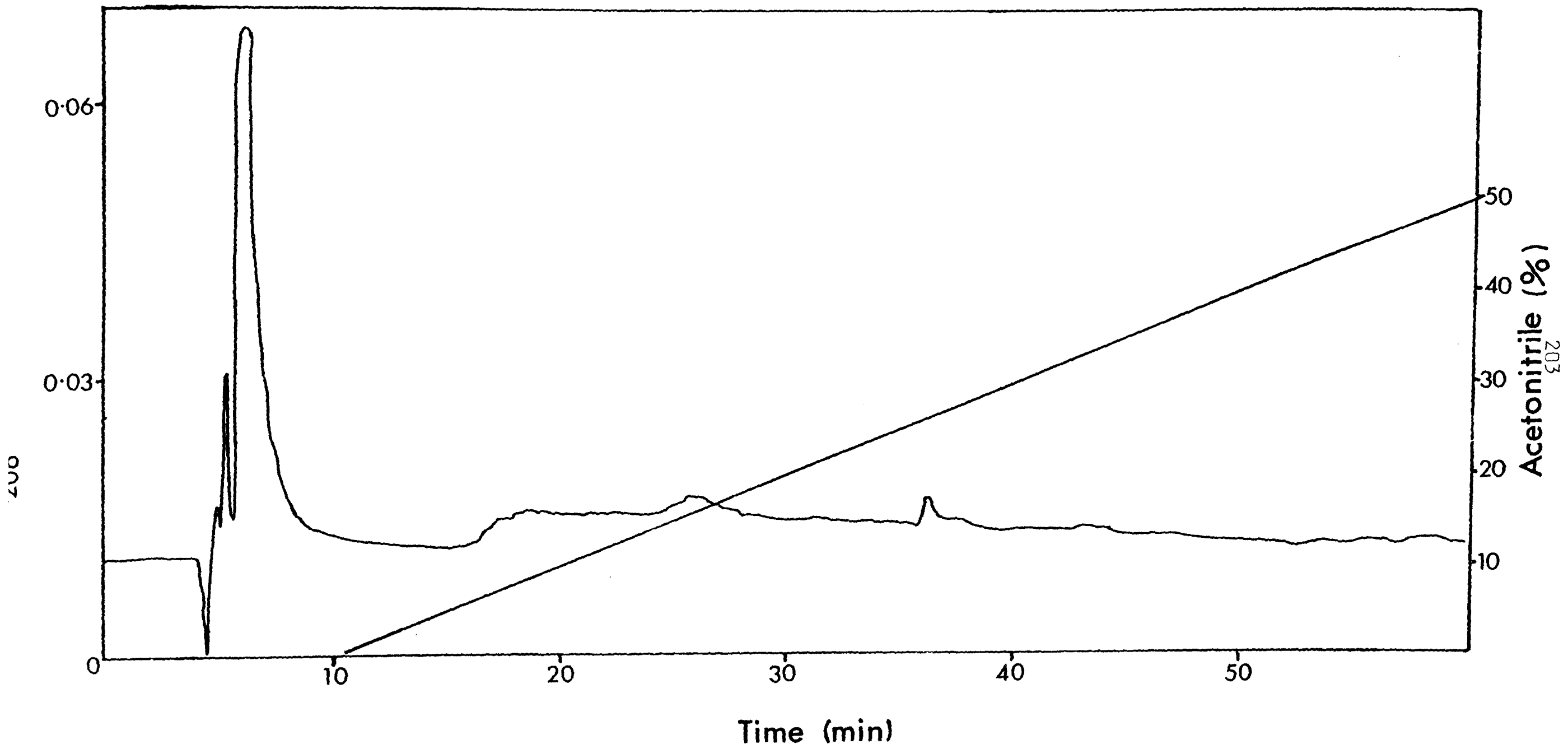


Figure 4.2.16. RP-HPLC chromatogram of EDTA inhibited enzyme (40 µg) incubated at 55°C for 30 min

were incubated at 37°C for 30 min in 50 mM Tris-HCl pH 8.0 in the presence of 10 mM $CaCl_2$, then incubated at 55°C for 5, 30 and 60 min. Preincubation of the enzyme at 37°C in the presence of $CaCl_2$ before heating at 55°C had no effect on the number of peaks observed (see Figures 4.2.15 and 4.2.17). Therefore, $CaCl_2$ did not protect the enzyme from autolysis at 55°C. Samples of the treated enzyme at 55°C for 30 min were incubated at 4°C overnight in the presence or absence of $CaCl_2$. There was no increase in the enzyme activity and no differences in the chromatograms before and after incubation at 4°C

4.2.14 Discussion

The purified protease secreted by P. fluorescens R8 was characterised. The enzyme had an optimum temperature for activity around 40°C. In this respect it is similar to most other proteases from Pseudomonas spp (Juan & Cazzalo, 1967; Alichanidis & Andrews, 1977; Patel et al., 1983). A rapid loss of activity at temperatures above 45°C was found. Heat stable proteases from Pseudomonas spp are characteristically unstable at temperatures between 50-60°C (Section 1.4.2.1). Pseudomonas aeruginosa protease, however, has been found to have an optimum temperature of 60°C (Morihara, 1963), which is high for most proteases of bacterial origin.

The enzyme in this study had an optimum pH around 7.5. It was active over a wide pH range. An optimum pH in the range 6.0-9.0 has been observed (Section 1.4.2.5) for proteases from Pseudomonas spp.

The R8 protease was similar to most Pseudomonas

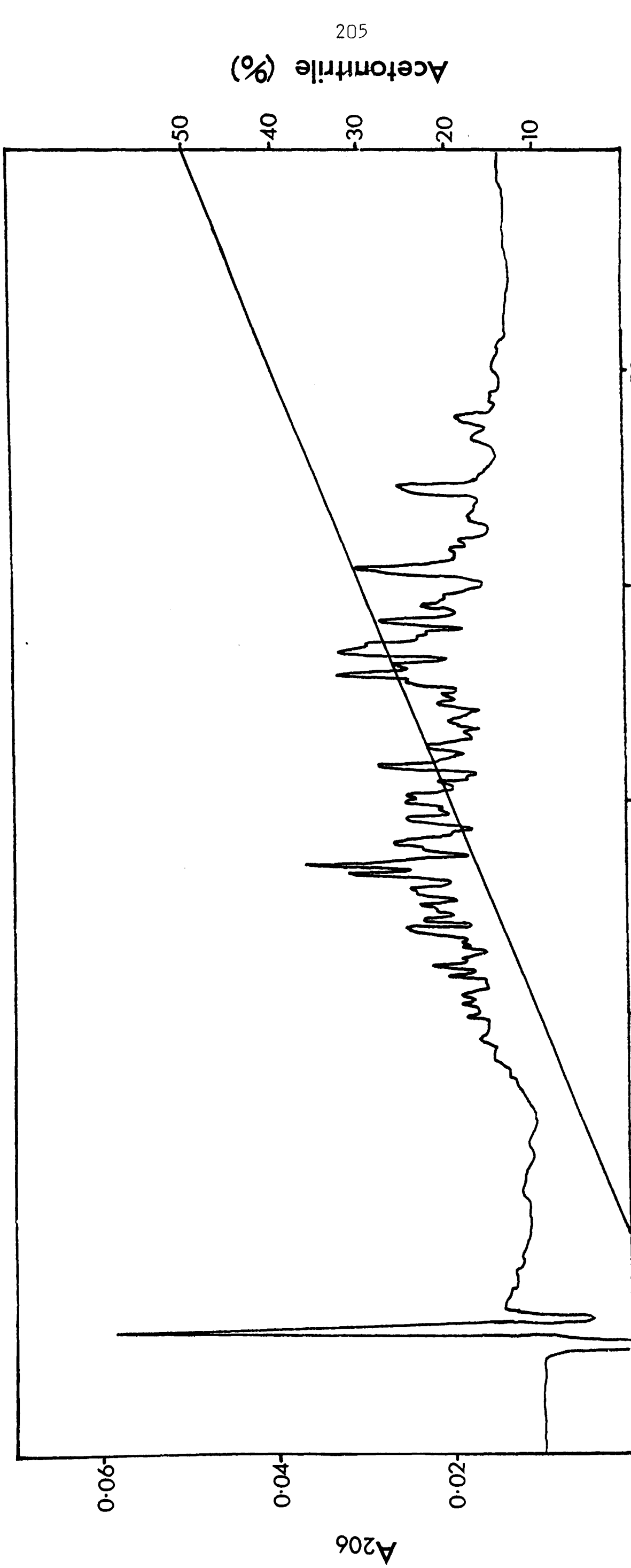


Figure 4.2.17. RP-HPLC chromatogram of enzyme (40 µg) preincubated at 37°C in the presence of $CaCl_2$ followed by incubation at 55°C for 30 min

proteases in that it is a metallo-enzyme. Two exceptions have been reported, one being the protease secreted by P. maltophilia which was a serine protease. However, this strain has recently been transferred to the Xanthomonas genus (Swings et al., 1983). The second example is the protease studied by Alichanidis & Andrews (1977), which was grouped into the thiol protease class of enzymes.

The enzyme in this study contained six atoms of calcium and two atoms of zinc per molecule. Many investigators have reported the presence of calcium and zinc in Pseudomonas spp proteases (Table 1.4.2.4). Calcium appears to be important in maintaining the integrity of the active site of the enzyme and in stabilising the native conformation of the molecule (Voordouw & Roche, 1974; Richardson, 1981). The presence of zinc has been reported to be essential for the activity of a number of bacterial extracellular proteases (Pangburn et al., 1976). The function of zinc is presumably catalytic, because it can restore the activity of an apoenzyme.

Results from studying the effect of inhibitors indicate that the enzyme was insensitive to sulphydryl group-blocking reagents and inhibitors of thiol proteases, therefore thiol groups were not involved in the active site of the enzyme. These results were confirmed by the amino acid composition. The enzyme was sensitive to o-phenanthroline, EDTA and EGTA. o-Phenanthroline was more inhibitory than EDTA probably because the enzyme had two atoms of zinc and eight atoms of calcium per molecule. Some investigators (Stepaniak et al., 1982b; Fairbairn & Law, 1986b)

have found that o-phenanthroline was less inhibitory than EDTA. However, the enzyme studied by Fairbairn & Law (1986b) contained only one atom of calcium and one atom of zinc. When the R8 enzyme was incubated against 1 mM EDTA 46% of activity remained. It was reported that metallo-neutral proteases are usually sensitive to EDTA at concentrations less than 1 mM (McConn, 1964; Morihara, 1974). The results obtained from investigating the effect of EDTA, optimum pH and the work done by Leadbeater et al. (1987) led to the conclusion that the protease secreted by P. fluorescens R8 was a metallo-alkaline protease.

TPCK which alkylates the active site histidine residue of chymotrypsin caused no inhibition and therefore it is probable that the enzyme did not have histidine in its active site. Pepstatin which inhibits pepsin, cathepsin D and other acid proteases (Whitaker, 1981) had no effect on the protease of P. fluorescens R8 which suggests that aspartic residues were also not involved in the active site. Phosphoramidon, synthesised by Streptomyces tanashiensis has been reported to be a specific inhibitor of metallo-proteases of microbial origin (Komiyama et al., 1975a,b), especially thermolysin and metallo-proteases from B. subtilis, Streptomyces griseus, P. aeruginosa and Streptococcus diacetylactis. Stepaniak et al. (1982b) found that phosphoramidon did not inhibit the metallo-protease secreted by P. fluorescens AFT 36. Gripon et al. (1980) reached the same conclusions and they suggested that sensitivity to phosphoramidon may be a simple way of sub-classifying metallo-proteases. Phosphoramidon in this

study had only a slight effect on the protease activity (96% activity remained), therefore the present result supports this suggestion. Most of the metal ions studied did not affect the enzyme activity. However, incubation with $ZnCl_2$, $NaCl_2$ and $CoCl_2$ has been found to result in some loss of activity. Hurley et al. (1963) found that there was no change in proteolytic activity in the enzyme from P. fluorescens due to the presence of metal ions.

The enzyme had high levels of aspartic acid and glycine; no cystine was found and it contained a minimal amount of methionine. The amino acid composition is very similar to that of other proteases from Pseudomonas spp (Mayerhofer et al., 1973; Richardson, 1981; Mitchell et al., 1986). The protease studied by Fairbairn & Law (1986b) appears to be a unique enzyme in that it is the only protease from Pseudomonas spp which has been reported to contain cysteine.

Attempts have been made to correlate the amino acid composition with heat stability of Pseudomonas proteases. The absence of disulphide bonds was noticed in these enzymes which provides a degree of flexibility to the primary structure (Bul & Breese, 1975). However, the heat stable protease studied by Fairbairn & Law (1986b) contained 0.8% cysteine. A second observation that may be related to thermal stability is the high content of low molecular weight residues (ie glycine and alanine); these could minimise steric hindrance in these enzymes (Richardson, 1981). However, Mitchell et al. (1986) found no difference in the level of these amino acids between

heat-stable and heat-labile proteases. Therefore the overall amino acid composition does not determine the heat stability of the protease. The difference in heat stabilities between pseudomonad proteases is probably due to the difference in amino acid sequence. Argos et al. (1979) studied the amino acid sequence of molecules of ferredoxin, lactate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase with different stabilities. It was found that Gly, Ser, Ser, Lys and Asp in the mesophilic enzymes were substituted by Ala, Ala, Thr, Arg and Glu, respectively in the thermophilic enzymes. These workers suggested that the thermal stability of the thermophilic enzymes was probably due to an additive effect of many substitutions made within the molecule.

The total carbohydrate content of P. fluorescens R8 protease was 0.12% and Dr I. Sutherland (personal communication) pointed out that the enzyme was not considered to be a glycoprotein because the total carbohydrate was very low (1 g of CHO would be present in 150,000 g of protein). Mitchell et al. (1986) however, using the same assay, classified the pseudomonads proteases which had 0.03-0.23% carbohydrate as glycoproteins. Fairbairn & Law (1986b) found that the protease secreted by P. fluorescens NCDO 2085 did not contain glucosamine or galactosamine and therefore it was not considered a glycoprotein.

The enzyme reported in this study was heat-stable having a D-value at 140°C of 1 min. It survived UHT treatment and high temperature short time pasteurisation (HTST) with 49 and 28% activity remaining respectively.

When the enzyme was heated at 140°C there was rapid loss on initial heating but then it levelled out producing biphasic heat-inactivation curves. The heat stable proteases from other Pseudomonas spp (Richardson, 1981; Stepaniak & Fox, 1983; Fairbairn & Law, 1986b) also exhibited biphasic heat inactivation curves in buffer. Stepaniak & Fox (1983) suggested that the stabilisation of a small proportion of the protease by Ca^{2+} to heat denaturation results in this biphasic curve. The heat stability of the protease studied by Barach et al. (1976a) was also enhanced in the presence of Ca^{2+}. However, the addition of 50 µM Ca^{2+} to the protease (1 µM) secreted by P. fluorescens NCD 2085 (Fairbairn & Law, 1986b) did not change the heat stability at 74°C. Furthermore Barach et al. (1976a) found that the rapid initial loss of the activity of Pseudomonas MC60 was eliminated by the addition of 0.5 mM Zn^{2+} to the sample. By contrast, Stepaniak & Fox (1983) did not find the same result when they studied the heat stability of the protease secreted by P. fluorescens AFT36.

The heat-stable proteases of Pseudomonas have been found to be unstable at temperatures between 55-60°C (Barach et al., 1978; Stepaniak & Fox, 1983; Fairbairn & Law, 1986b). The protease in this study was unstable at 55°C either in the presence or absence of $CaCl_2$. Only 4% of the initial activity remained after heating the enzyme at 55°C for 5 min and there was no increase in activity after incubating the enzyme at 4°C overnight. Barach et al. (1976b) found that 10% of the initial activity of pseudomonads proteases remained after heating at 55°C for

10 min. Barach et al. (1978) and Richardson (1981) reported that the inactivation of heat stable proteases at 55°C was due to autolysis. This was confirmed from the chromatograms obtained after using RP-HPLC. More than 25 peaks were observed. The enzyme protein itself was hydrophilic and it was not retained on the C8 RP-column. This meant it was not possible to monitor the disappearance of the enzyme as the peak caused by the digestion buffer completely masked the enzyme peak. In order to follow the disappearance of the enzyme by this method it would have been necessary to either use a RP-column on which the enzyme was retained or to carry out further sample preparation steps to remove digestion buffer. Further manipulation of the samples may have lead to further autolysis and considerably increased the time taken to achieve results. The major advantage of RP-HPLC over PAGE for example is its speed and sensitivity. Samples were loaded onto RP-HPLC to rapidly enable the determination of autolysis. The use of other RP-HPLC columns would have necessitated further method development which can be time-consuming. Therefore RP-HPLC was only used qualitatively to study the autolysis at 55°C.

Inhibition of the enzyme (using EDTA) prevented autolysis and one peak only was observed. These results are in line with the autolytic degradation observed on PAGE when active enzyme was used (Sections 4.1.1 and 4.1.3). The mechanism of low temperature activation is not fully understood. Stepaniak & Fox (1983) studied the inactivation at 55°C of P. fluorescens AFT36 protease and they suggested that the autolysis at this temperature

appears to be due to intramolecular autolysis. On the other hand Diermayr et al. (1987) investigated the mechanism of inactivation of P. fluorescens biotype I strain 112 protease and suggested that the LTI effect could be due to intermolecular rather that intramolecular autolysis. The mechanism of the LTI may vary with enzymes from different species of bacteria. Four of the eight enzymes studied by Mitchell et al. (1986) showed little decrease in proteolytic activity due to LTI.

The protease in this study degraded milk proteins as well as other proteins like bovine serum albumin and cytochrome c. The enzyme was more active against whole casein than any of its fractions. β-Casein was the most susceptible casein fraction to the enzyme action. Knaut et al. (1965) and Purschell & Pollack (1972) also found that β-casein was degraded to a greater extent than α-casein by proteases from psychrotrophic bacteria. However, Yan et al. (1985) revealed that α_s-casein was the most susceptible casein fraction and k-casein was the least susceptible to the action of protease secreted by Pseudomonas fluorescens LY13. Patel et al. (1983) studied the breakdown of casein, its fractions and some other proteins by six proteases from different Pseudomonas spp and concluded that the proteases differed in their substrate specificities. Some were found to prefer α-casein as a substrate and others showed higher activity in the presence of soluble casein, which is a mixture of other caseins. Guamis et al. (1987) studied the breakdown of milk proteins by different strains of psychrotrophic bacteria. They found that P. fluorescens degraded

all milk proteins after 3 d at 7°C while Cytophaga spp degraded mainly the caseins after 21 d at 7°C.

The R8 protease attacked β-lactoglobulin when the period of incubation was extended and the substrate was preincubated in buffer at pH 8 for 60 min. Gebre-Egziabher et al. (1980b) studied the hydrolysis of milk proteins by six psychrotrophic Pseudomonas. They found that most of the strains required extended incubation periods for breakdown of whey proteins than they needed to hydrolyse k- and β-casein. Adams et al. (1976) also revealed that Pseudomonas spp degraded α-lactalbumin and β-lactoglobulin, the breakdown ranging from 0 to 39% after 13 days at 5°C depending on the species and strain. On the other hand some workers (Cousin & Marth, 1977a; Law et al., 1977) found that whey proteins were not hydrolysed by the enzymes they studied. Fox (1982) also reported that β-lacto-globulin and α-lactalbumin were resistant in the native form to the action of highly active non-specific proteases.

The enzyme reported in this study did not attack trypsin, chymotrypsin and estrase substrates. These findings are in good agreement with the results obtained by Richardson (1981). Mitchell et al. (1986) also studied the specificity of proteases from six strains of Pseudomonas fluorescens and two strains of Serratia marcescens and reported that all the proteases studied appeared to have a narrow specificity for low molecular weight peptide sub-strates. The specificity of R8 protease towards insulin β-chain was studied. The enzyme had broad specificity; it hydrolysed bonds containing bulky, hydrophobic, small and

hydrophilic amino acids (Leadbeater et al., 1987). The protease of P. fluorescens R8 was found to have a molecular weight of 45 ± 1 K daltons. Most of extracellular proteases secreted by Pseudomonas spp are of the size 40-50 K daltons (Table 1.4.2.4).

4.2.15 Summary

The heat stable extracellular protease (peak A) of Pseudomonas fluorescens R8 was characterised. It was found to be a metallo-alkaline protease of 45 K daltons which contained calcium and zinc. The enzyme had an optimum temperature around 40°C and optimum pH ≈ 7.5 and was sensitive to o-phenanthroline, EDTA and EGTA. It was more sensitive to o-phenanthroline than EDTA and EGTA. It was found to contain higher levels of aspartic acid and glycine, but did not contain cysteine. The total carbohydrate content was very low, therefore the enzyme was not considered to be a glycoprotein. Peak B was found to have the same molecular weight as peak A and the immunochemical analysis showed that they were identical.

The enzyme was heat stable, the D-value at 140°C was 1 min and 28% of the original activity remained after heating the enzyme at 74°C for 17 sec. The protease was inactivated by heating at 55°C in buffer in the absence or presence of $CaCl_2$. Casein, its fractions, bovine serum albumin and cytochrome c were attacked by the enzyme. β-Lactoglobulin was also susceptible to hydrolysis by R8 protease.

CHAPTER 5

FINAL DISCUSSION

5.1 Identification of a Pseudomonas spp strain from raw milk, production, purification and characterisation of its protease

Growth and production of extracellular enzymes by psychrotrophic bacteria in refrigerated dairy products has stimulated research on the metabolic activity of these bacteria. Most psychrotrophic bacteria secrete extracellular proteases, lipases and phospholipases. These bacteria contaminate milk from soil, water and poorly cleaned dairy equipment (Cousin, 1982). The aim of the work presented in Section 3.1 was to isolate and identify proteolytic psychrotrophic bacteria from some of the East of Scotland farms. Gram-positive and Gram-negative protease-producing bacteria were isolated and identified. The Gram-positive proteolytic psychrotrophic bacteria were found to belong to four genera: Bacillus, Micrococcus, Corynebacterium and Staphylococcus. The Gram-negative genera were Pseudomonas, Acinetobacter, Flavobacterium and Cytophaga. Flavobacterium and Pseudomonas were the two main genera of Gram-negative bacteria. They represented 48.8 and 34% of the total protease-producing bacteria. Although the majority of psychrotrophic Gram-negative rods isolated from raw milk belonged to Pseudomonas spp (Richard, 1981; Malik & Mathur, 1983), it was found in the present study that Pseudomonas spp were less numerous than Flavobacterium spp. The reason for that was two of the tested milk samples were highly contaminated with Flavobacterium spp. Pseudomonas fluorescens represented 42.9% of the Pseudomonas spp isolated. This is in good agreement with previous work by

Law (1979), who showed that P. fluorescens was the most common species isolated from raw milk. Pseudomonas fluorescens R8 showed high proteolytic activity when grown on agar medium supplemented with skim milk or when grown on broth medium; this strain was therefore chosen to investigate the induction of the protease (Section 3.2).

Production of extracellular enzymes can be classified as inducible, partially constitutive and constitutive depending on the organism and the enzyme secreted (Priest, 1983, 1984). The enzyme is described as inducible when the presence of the substrate or its derivatives dramatically increase the rate of synthesis of the enzyme. The extracellular enzyme could be described as partially constitutive if the basal level of the enzyme varies considerably from a barely detectable activity to a relatively high level. If the rate of the enzyme biosynthesis is constant irrespective of the presence or the absence of the substrate in the medium the enzyme is considered constitutive. In the light of the results obtained from Section 3.2, the protease secreted by strain R8 could be described as a constitutive protease because the biosynthesis of the enzyme was not greatly affected by the presence or absence of proteins or protein degradation products in the growth medium. Only two species of Pseudomonas have been reported to produce constitutive proteases (Keen & Williams, 1967; Boethling, 1975) and most porteases from Pseudomonas spp were found to be inducible. Pseudomonas fluorescens NCDO 2085 was not able to produce an extracellular protease when grown in the presence of inorganic nitrogen (Fairbairn &

Law, 1987). Proteins and peptides with molecular weight of 1000-60 000 have been found to stimulate the biosynthesis of extracellular proteases produced by Pseudomonas spp (McKellar, 1982; McKellar & Cholette, 1984). α_{S2}-casein stimulated the production of protease by P. fluorescens NCDO 2085 more than the other casein fractions (Fairbairn & Law, 1987). Amino acids were found to be either protease inducers or repressors depending upon the type of amino acid involved and the metabolic character of the micro-organism (Jensen et al., 1980a; McKellar, 1982).

The effect of various carbon sources on the production of the protease by strain R8 was investigated. The protease produced (EU growth^{-1}) in glucose and glutamate basal media was higher than when succinate, malate, pyruvate or glutamate basal media were tested. Glucose did not repress the biosynthesis of the protease by Pseudomonas fluorescens R8; this is further evidence that the enzyme is constitutive. It has been proposed that glucose and other easily utilisable carbon sources are metabolised rapidly producing high levels of catabolite intermediates which cause repression of the synthesis of inducible enzymes (Magasanik, 1961). Fairbairn & Law (1987) found that citrate repressed the enzyme production by P. fluorescens NCDO 2085. It is perhaps surprising that P. fluorescens isolated from raw milk did not utilise lactose, the most abundant carbohydrate in milk.

Although nitrogen and energy sources were found to be the most influential factors in the biosynthesis of extracellular proteases by Pseudomonas spp, mineral ions were

also found to affect the production of protease. The effect of calcium on the protease production in glutamate and glucose basal medium was investigated. In glutamate basal medium the presence of $CaCl_2$ increased the enzyme produced (EU growth^{-1}) 1.4 fold. Calcium in glucose basal medium increased enzyme production about seven times over control medium. However, in both media growth was not affected by the presence or absence of $CaCl_2$. These experiments do not distinguish between the effect of calcium ions on the protease synthesis or activity. This can only be done using an assay specifically for protease protein (see next page). It would be interesting to study the effect of other metal ions such as Zn^{2+} or Mg^{2+} on the enzyme production using different carbon and nitrogen sources. Calcium ions have been reported to affect the stability and activity of many extracellular proteases secreted by Pseudomonas spp (Amrute & Corpe, 1978). Presence of $CaCl_2$ in the growth medium increased the enzyme activity between 5-9 fold (Amrute & Corpe, 1978; Fairbairn & Law, 1987). In addition the study on the effect of $CaCl_2$ in the growth medium carried out by McKellar & Cholette (1986) revealed that calcium may be required for the formation of an active protease from newly synthesised polypeptide chain. When $CaCl_2$ was omitted from the medium an irreversibly inactive "precursor" (12-14K daltons) was formed.

The effect of other ions such as phosphate, ammonium and sodium on protease production have been studied previously. Orthophosphate (5 mM) was required for the

maximum production of the protease secreted by P. fluorescens (McKellar & Cholette, 1984). Ammonium ions were found to inhibit the production of protease and other extracellular enzymes by P. aeruginosa (Kessler & Safrin, 1983).

In the present work protease activity (EU growth^{-1}) was detected at the beginning of growth. By contrast, the protease secreted by Pseudomonas fluorescens NCDO 2085 did not produce protease until the end of the exponential phase/beginning of the stationary phase (Fairbairn & Law, 1987). Very little is known about the pattern of production of extracellular enzymes as measured by protein level rather than enzymic activity. In the present study, quantitation of the enzyme protein was done by the application of rocket immunoelectrophoresis. By carrying out this technique it was found that enzyme protein (EP growth^{-1}) in glucose basal medium containing $CaCl_2$ was about 9.2 fold higher than in the absence of $CaCl_2$. Enzyme activity per growth was about 6 fold higher in the presence of $CaCl_2$ than when it was omitted. These results and the results obtained when glutamate basal medium was used confirm that $CaCl_2$ affects the synthesis and the activity of the extracellular protease secreted by Pseudomonas fluorescens R8. Unfortunately the amount of antisera raised was not sufficient to investigate the relationship between enzyme protein and enzyme activity using basal medium (supplemented with different carbon sources) and complex medium. The enzyme protein was detected at the beginning of growth in the presence and absence of $CaCl_2$. These findings are

not in agreement with the results obtained by McKellar & Cholette (1986) who revealed that in the absence of $CaCl_2$ an irreversibly inactive "precursor" was synthesised. It would be interesting to determine the molecular weight of the enzyme produced in basal medium in the presence and absence of $CaCl_2$.

Pseudomonas fluorescens R8 was grown in trypticase soy broth supplemented with 1% skim milk. The enzyme produced was a major component in the medium. Purification was achieved in three stages: ultrafiltration, ammonium sulphate precipitation and ion exchange chromatography. Two peaks were obtained after ion exchange chromatography, peak (A) and peak (B); they both have the same molecular weight and immunochemical identity has also been found for the two peaks. Probably they differ very slightly in amino acid composition, as they eluted differently during ion exchange chromatography. There was little time to investigate the differences between these two peaks. Peak A was studied in detail because it contained about 60% of the total activity while peak B contained about 15%. It was difficult to locate the enzyme on native gels (zymogram staining). There was no distinct clear zone around the enzyme protein. Instead a smeary clear zone was observed. The appearance of this smeary zone was due to aggregation. This problem may be solved by running an SDS-PAGE at 4°C without boiling the enzyme samples.

Although Pseudomonas spp are heat labile bacteria, most of them secrete heat stable proteases which can withstand UHT treatment and cause problems like the

appearance of off-flavour in milk and its products. The R8 protease was found to be a heat stable protease with a D-value at 140°C of 1 min. The heat stabilities of some extracellular proteases produced by Pseudomonas spp are summarised in Table 1.4.2.1. The heat stability of these proteases may be due to structural flexibility as a result of the lack of cysteine and the interchange of divalent cations. After thermal denaturation this flexibility may allow rapid renaturation when the temperature is subsequently lowered.

It would be very interesting to study in more detail the heat stability of proteases secreted by Pseudomonas spp and to investigate the difference between these enzymes and heat labile proteases; studying amino acid composition did not differentiate between heat stable and heat labile enzymes (Mitchell et al., 1986). Therefore, investigating the amino acid sequence of heat stable proteases probably would lead to better understanding of the thermostability of these enzymes and possibly to control of their activity.

Although most extracellular proteases produced by Pseudomonas spp are heat stable to UHT treatment, some of them have been found to be unstable at 55°C. The effect of treating the enzyme from P. fluorescens R8 at this temperature was investigated (Section 4.2). The inactivation at 55°C was studied using reversed-phase high performance liquid chromatography (RP-HPLC). This method was rapid, sensitive and small fragments could be detected; it was also possible to analyse samples immediately after the heat treatment. This technique has many advantages compared to

other conventional methods. The time for analysis was very short so preventing any changes in the sample. The results showed that the protease in this study was unstable at 55°C and a large number of peaks were detected after treatment at 55°C for 5 min. Remaining activity was only 4%. Inhibition of the enzyme prior to treatment at 55°C (using EDTA) prevented autolysis and one peak only was detected. This suggests that the low temperature inactivation is caused by autolysis. This hypothesis has been considered previously by Stepaniak & Fox (1983) and Diermayr et al. (1987). The mechanism of inactivation at 55°C was thought to be either due to intramolecular autolysis (Stepaniak & Fox, 1983) or intermolecular autolysis (Diermayr et al., 1987).

Some biological characteristics of the protease were investigated (Table 5.1). The specificity of R8 protease towards milk proteins and other proteins such as bovine serum albumin and cytochrome c was studied (Section 4.3). The enzyme attacked β-, α_{S1}- and α_{S2}-caseins but was more active against whole casein than its fractions. Degradation of β-lactoglobulin was investigated using RP-HPLC. More than 20 bands were detected. The specificity of this enzyme against insulin β-chain has also been studied in this laboratory (Leadbeater et al., 1987). It hydrolysed bonds which contained bulky, hydrophobic, small and hydrophilic amino acids. The results obtained from studying the effect of various inhibitors and substrate specificity demonstrated that R8 protease could be classified as an alkaline metallo-protease. Limited information is avail-

Table 5.1 Physicochemical properties of an extracellular protease (peak A) produced by P. fluorescens R8

Optimum temperature	40°C
Optimum pH	≈ 7.5
Molecular weight	45 ± 1 K daltons
Average hydrophobicity	0.98 K cal residue^{-1}
Metal content	6 Ca 2 Zn
Inhibitors	o-phenanthrolein, EDTA, EGTA
D-value (140°C)	1 min

able about substrate specificity of Gram-negative bacterial proteases. Generally speaking, metallo-proteases are classified into neutral and alkaline metallo-proteases. The first group was found to hydrolyse basic and bulky or hydrophobic amino acids at the amino side of the splitting bond (Morihara, 1974; Fairbairn & Law, 1986a), while the second group showed no regularity in hydrolytic activity.

Pseudomonas fluorescens R8 degraded casein, its fractions and β-lactoglobulin. Hydrophobic peptides, in particular, bitter peptides were isolated, but not identified (Leadbeater et al., 1987). The action of proteases from Pseudomonas spp in heat treated milk and its products led to the appearance of bitter flavour (Cousin, 1982). Degradation of milk proteins by these enzymes causes off-flavour and off-odours and may produce inferior products manufactured from this milk. β-casein was found to be the main source of bitter peptides in milk and its products (Visser et al., 1983). However, some bitter peptides have also been isolated from α_{S1}-casein (Richardson & Creamer, 1973).

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