THE SPECIFICITY OF INTRACELLULAR PROTEIN DEGRADATION IN BACTERIAL CELLS

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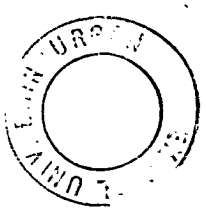
A Thesis Presented for
the Degree of
Ph.D.

Department of Molecular Biology
University of Edinburgh

January 1989



To Gill

Abstract

The *Escherichia coli* ATP-dependent protease La was purified from an over-expressing strain. The enzyme was judged to be 90% pure and to be free from contamination by non ATP-dependent proteases. The sequence of the first ten amino acids at the N terminus of protease La were in agreement with that predicted by the nucleotide sequence of the *lon* gene. Optimum conditions were determined and used in experiments to investigate the selectivity of the enzyme. Protease La was found to be highly selective *in vitro*; three proteins which are extremely sensitive to proteolysis *in vitro* were not degraded, and only two out of fourteen native protein preparations were digested by the protease. Several peptide bonds of radiolabelled proteins were hydrolysed by protease La. Non-hydrolysable analogues of ATP did not support digestion of radiolabelled proteins by protease La. The effect of DNA on the ATPase and proteolytic activities of protease La was investigated. DNA was found to stimulate, inhibit or have no effect on proteolytic activity depending upon the protein substrate used in the assays. The effect of DNA on ATPase activity measured in the presence of a protein substrate appears to correlate with its effect on the digestion of that substrate.

Acknowledgements

These miserable words fail to express my sentiments but they are all I have.

I would like to thank my supervisor Professor R.P. Ambler for providing the opportunity and means to pursue this project, and for advice at critical moments. I must also thank Richard for performing amino acid analyses.

I am greatly indebted to Margaret Daniel for instructing me in HVPE and for operating the FPLC, and to Tam Bruce for growing cells. I must thank the excellent support staff for providing media and clean glassware, especially Ruby Docherty and Joan Smail. I am indebted to Wel-Met for performing the amino acid sequence analysis.

For transforming pages of illegible scribbles into this well laid out document I must thank Jo and Ian Oliver. In this respect I must also give thanks to Annie Wilson and Gill Cowan. I thank Graham Brown for the photographs.

I am very grateful to Daniela, Joan, Margaret and Gudmundur for their help, support and interest during the past four years. Better friends one could not hope to find.

I am indebted to the SERC for a research studentship and to Gill Cowan for keeping me in peanut butter during the last year.

Finally, I owe an immense debt of gratitude to my wife for her understanding and tolerance during the last three years. She was the light at the end of a long, dark tunnel.

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Abbreviations

A	absorbance
ADP	adenosine 5′-diphosphate
AMP	adenosine 5′-monophosphate
AMP-PCP	β-γ-methyleneadenosine 5′-triphosphate
AMP-PNP	adenyl-5′-ylimidodiphosphate
AMPS	ammonium persulphate
ATP	adenosine 5′-triphosphate
BICINE	N,N-bis(2-hydroxyethyl)glycine
BSA	bovine serum albumin
cpm	counts per minute
DEAE	diethylaminoethyl
dimethyl POPOP	1,4-di-2-(4-methyl-5-phenyloxazolyl) benzene
DNA	deoxyribonucleic acid
DNP	dinitrophenyl
dsDNA	double-stranded DNA
DTT	dithiothreitol
E.	*Escherichia*
EDTA	diaminoethanetetraacetic acid
FPLC	fast protein liquid chromatography
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
g_n	standard acceleration due to gravity
HEPES	N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid
HVPE	high voltage paper electrophoresis
kb	kilobase
K_m	Michaelis constant
M_r	molecular weight
Ox.	oxidized
PAGE	polyacrylamide gel electrophoresis
PPO	2,5-diphenyloxazole
Q	quaternary aminoethyl
RNA	ribonucleic acid
rpm	revolutions per minute
S.	*Staphylococcus*
S.D.	standard deviation
SDS	sodium dodecyl sulphate

ssDNA	single-stranded DNA
SSC	standard saline citrate
T^c	tetracycline
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane
U.V.	ultra-violet
v:v	volume by volume
w:v	weight by volume

CHAPTER 1

Introduction

1.1 BACKGROUND

Life is a steady state. As proteins, nucleic acids and other cell components are synthesized and cells multiply, other molecules are degraded, and other cells die. This process of turnover goes on within healthy cells, and is selective and under stringent control.

Bacteria have the capacity to hydrolyse the peptide bonds of intracellular proteins. It is important to differentiate between the hydrolysis of a limited number of peptide bonds (proteolytic modification) and the hydrolysis of all of the peptide bonds of a protein (protein degradation). These processes involving peptide bond cleavage have totally different functions: proteolytic modification alters the properties of a protein, whereas protein degradation results in the loss of a protein and the recycling of its constituents.

1.2 INTRACELLULAR PROTEIN DEGRADATION IN BACTERIAL CELLS

The appearance of Schoenheimer's "The Dynamic State of Body Constituents" (1942) confirmed the belief that there is a continuous turnover of the components that make up living organisms. It was therefore surprising that experiments designed to find out whether β-galactosidase induction in *E. coli* involves *de novo* synthesis or the activation of a precursor protein, suggested that proteins in bacteria are stable, or are degraded extremely slowly (Hogness *et al.*, 1955; Rotman and Spiegelman, 1954). However, conditions that allowed detection of protein degradation in *E. coli* were found a few years later. Mandelstam (1958) discovered that although protein degradation in growing cells of *E. coli* was difficult to detect (<1% labelled protein was degraded to amino acids per hour), starvation for nitrogen led to an easily measured degradation rate (about 5% h^{-1}). Furthermore, a recent experiment revealed that in glucose-limited chemostat-grown *E. coli* (doubling time 14.5h), about 60% of label incorporated into proteins during a 1h pulse was released as acid-soluble radioactivity during the subsequent growth through about five generations (St. John *et al.*, 1979). For technical reasons, measurements had to stop after 72h but it appears that most, if not all, of the proteins in growing bacteria are subject to degradation, and an earlier estimate that only 30% of protein in bacteria is subject to degradation under any conditions (Pine, 1973) is probably incorrect.

Estimates of the rate of protein degradation in growing bacterial cells depend upon how experimental measurements are made. By growing *E. coli* for several generations in medium containing a radioactive amino acid to enable a steady state of

labelling to be realized, Mandelstam (1958) and Willetts (1967b) reported a very low rate of protein degradation: about 1% labelled protein was degraded to amino acids per hour. In contrast, measuring the degradation of proteins labelled by a short pulse of radioactive amino acid gave a very much greater rate of degradation (Pine, 1970). At least 5% of the label incorporated in a 7 second pulse was released during a 45 second chase period. Following this rapid degradation of pulse-labelled protein, the rate fell steadily with continued growth to give an average value of 2.5% pulse-labelled protein degraded to amino acids per hour, which is in better agreement with the values reported by Mandelstam (1958) and Willetts (1967b).

Discrepancies in the rate of protein degradation revealed by the different labelling protocols indicates that proteins in bacteria are digested at very different rates. Exposure of cells to radioactive amino acids for several generations preferentially labels proteins which are degraded slowly (the stable fraction), whereas exposure to a pulse of radioactive amino acids labels proteins which are rapidly digested (the labile fraction). Most of the proteins in a bacterial cell are thought to belong to the stable fraction, whilst only 2–7% of total cell protein may constitute the labile fraction (Nath and Koch, 1970). Thus it appears that growing cells of *E. coli* have the ability to carry out selective protein degradation. Such an ability was clearly demonstrated by Pine (1967) who showed that *E. coli* can selectively degrade structurally altered (abnormal) proteins during growth. Abnormal proteins shown to be rapidly degraded by *E. coli* are of several types:

1. Incomplete polypeptides resulting from nonsense mutations (Goldschmidt, 1970), deletion mutations (Platt *et al.*, 1970), internal initiation of translation (Apte *et al.*, 1975), or incorporation of puromycin (Goldberg, 1972; Pine, 1967).
2. Full length polypeptides with structural defects resulting from missense mutations (Berquist and Truman, 1978; Zipser and Bhavsar, 1976), incorporation of amino acid analogues (Goldberg 1972; Pine, 1967), or mistranslation caused either by *ram* (ribosomal ambiguity) mutations or missense suppressors (Goldberg, 1972).
3. Full length polypeptides that fail to assemble into a multimeric structure (Hayward *et al.*, 1974; Dennis, 1974).
4. Products of eukaryotic genes expressed in *E. coli* (Young and Davis, 1983).

Abnormal proteins accumulate preferentially within rapidly sedimenting cell fractions prior to hydrolysis (Prouty and Goldberg, 1972; Prouty *et al.*, 1975). This has often facilitated the purification of foreign gene products expressed in *E. coli* (see

Marston, 1986). The structures sedimenting at 10–20,000g_n correspond to the dense, intracellular inclusions (granules) observed in electron micrographs of *E. coli* grown in medium containing amino acid analogues (Schachtele *et al.*, 1968; Rabinovitz *et al.*, 1969). Further study of these granules revealed that they are not degradative organelles comparable to lysosomes since they are not membrane bound and they do not contain hydrolytic enzymes (Prouty *et al.*, 1975). Instead, they resemble amorphous precipitates of denatured protein (Prouty *et al.*, 1975). Granule formation may involve the spontaneous aggregation of highly abnormal polypeptides and occurs in cells containing large amounts of these polypeptides (Prouty *et al.*, 1975; Goldberg and St. John, 1976).

The role of granule formation is not understood. Possibly the aggregation of abnormal polypeptides promotes their recognition by the cell's degradative machinery. However, formation of large granules is not an essential step in the degradative process since many aberrant polypeptides which do not accumulate in such structures are rapidly hydrolysed (Goldberg and St. John, 1976). Alternatively, granule formation may simply reflect that the amount of abnormal polypeptide within the bacterium is greater than the degradative capacity of the cell: when the proteolytic machinery becomes saturated the excess aggregates into granules (Goldberg and St. John, 1976).

Although labelling cells with radioactive amino acids for varying lengths of time revealed that the rates of degradation of proteins are dissimilar, this approach gave little information about the rate of degradation of individual protein species. Since only a few protein species comprise most of the soluble protein in *E. coli* (50 proteins make up about 75% of cell protein; O'Farrell, 1975), a large proportion of the protein species could be rapidly digested without significantly affecting the overall rate of protein degradation. Consequently, Mosteller *et al.* (1980) measured the rate of degradation of individual protein species in growing cells of *E. coli*. Using two-dimensional electrophoresis to resolve labelled proteins, 47 out of 184 proteins were found to be digested with estimated half-lives ranging between 2 and 23 hours. This study provided evidence that *E. coli* has the ability to carry out selective protein degradation. However, this experiment could not distinguish proteins which are degraded from those that are modified and therefore have altered positions on the two-dimensional gel. Surprisingly, in a similar experiment using a different labelling procedure only 3 out of about 250 proteins were found to be digested with estimated half-lives of 4.5 hours or less. This discrepancy is not understood at present. Unfortunately, the rates of degradation of only a few proteins of *E. coli* have been measured directly. Many of the proteins studied were found to be stable during the

period of measurement (β-galactosidase, Goldschmidt, 1970; serine deaminase and alkaline phosphatase, Willetts, 1967a; acyl carrier protein, Powell *et al.*, 1973, elongation factor G and initiation factor F_2, Krauss and Leder, 1975; β and β'-subunits of RNA polymerase, Kirschbaum *et al.*, 1975; *lac* repressor, Platt *et al.*, 1970). However, several λ proteins (*N, O, xis* and *c*II products, Gottesman *et al.*, 1981 and *c*I product; Roberts and Roberts, 1975) and *E. coli* proteins (LexA, Little *et al.*, 1980; SulA, Mizusawa and Gottesman, 1983; UmuD and C, Marsh and Walker, 1985; HtpR, Grossman *et al.*, 1985; RcsA, Torres-Cabassa and Gottesman, 1987) are rapidly degraded with estimated half lives ranging between 2 and 7 minutes. The *c*I and *lexA* products are rapidly digested only after U.V. irradiation.

1.3 PHYSIOLOGICAL SIGNIFICANCE OF INTRACELLULAR PROTEIN DEGRADATION

Early ideas regarded intracellular protein degradation in bacteria solely as an important response to starvation (Mandelstam, 1960; 1963). Increased protein degradation upon starvation provides a source of amino acids for the synthesis of proteins essential to survival under the prevailing conditions (Mandelstam, 1958). Further study suggested that the absolute rate of peptide bond hydrolysis does not increase upon starvation: proteins which are relatively resistant to digestion in growing cells become available for breakdown (Pine, 1966). The identities of these proteins are unknown. However, it is tempting to speculate that starving cells selectively degrade growth-related proteins such as enzymes for DNA or protein synthesis. These proteins may be unnecessary or even potentially harmful to non-growing cells. Currently, there is little evidence available to support such selectivity (Miller, 1987).

Changes in the susceptibility of proteins to degradation, or alterations in the proteolytic machinery could be the cause of increased protein degradation during starvation (Goldberg and St. John, 1976; Miller, 1987). Binding to substrates has frequently been shown to protect enzymes from proteolysis *in vitro*; it is possible, therefore, that certain proteins become more susceptible to digestion during starvation because their substrates are present at reduced levels (Miller, 1987). However, the necessary selectivity inferred by this hypothesis has not been reported. Furthermore, the susceptibilities to degradation by several endopeptidases *in vitro* of bulk protein from growing, and from non-growing cells do not differ (Goldberg and St. John, 1976). The proposal that starvation leads to synthesis of additional proteases lacks experimental support (Miller, 1987); similarly, factors which modulate the pre-existing proteolytic machinery during starvation have not been discovered (Goldberg and St. John, 1976; Miller, 1987). A novel explanation for the increased degradation of

normally stable proteins during starvation was suggested by Miller (1987). Starvation leads to an interruption in the substantial flow of preferred substrates through the proteolytic machinery because the synthesis of these protein substrates has ceased. This enables the proteases to degrade a different group of proteins. At present, there is insufficient experimental evidence to substantiate any of these hypotheses. Increased knowledge from future investigations may provide such support or lead to alternative explanations.

Extensive proteolysis of newly synthesized proteins has been shown to occur in growing cultures of *E. coli* (Pine, 1970). Measurement of the digestion of protein labelled by a very short pulse of radioactive amino acid revealed that a substantial fraction (at least 5%) was rapidly degraded. A different estimate of the size of this rapidly digested fraction (at least 20%) was deduced from an experiment involving a peptidase deficient strain of *Salmonella typhimurium* (Yen *et al.*, 1980). These experiments suggest that many proteins are degraded before they have displayed activity. This phenomenon appears wasteful in terms of energy expenditure: both synthesis and hydrolysis of peptide bonds require metabolic energy. However, since the rapid degradation of newly synthesized polypeptides is such a major metabolic process (Pine, 1970; Yen *et al.*, 1980) it must be physiologically significant. Continued investigation has begun to clarify the role of this process.

Recently, some of the constituents of the rapidly digested fraction have been identified. Signal peptides cleaved from exported proteins and attenuator peptides produced by translation of regulatory sequences are quickly hydrolysed (Hussain *et al.*, 1982; Miozzari and Yanofsky, 1978), possibly as an economy measure. Several regulatory proteins of *E. coli* (such as LexA, Little *et al.*, 1980; SulA, Mizusawa and Gottesman, 1983; UmuD and C, Marsh and Walker, 1985; HtpR, Grossman *et al.*, 1985; and RcsA; Torres-Cabassa and Gottesman, 1987) are also highly sensitive to proteolysis; presumably to allow the cell to respond swiftly to changing conditions. Aberrant polypeptides (which have been shown to be rapidly digested *in vivo*) generated by various kinds of translational errors, such as out-of-phase initiation of translation, translational frameshifting, readthrough of termination signals, or premature termination of translation, may also contribute to the rapidly degraded fraction (Miller, 1987). Aberrant polypeptides arising from the premature release of peptidyl tRNA during translation (Menninger, 1977) and from premature termination of translation (Manley, 1978) have been shown to occur. Kurland and Ehrenberg (1984) argue that selective forces have produced a translational apparatus with less than maximum accuracy. Thus it seems likely that a major role of the cell's proteolytic machinery is

to rapidly destroy aberrant polypeptides, so that the cell does not become corrupted by the build-up of defective proteins. In this respect, proteolysis may identify a proof-reading function which is more rapid or less energy expensive than increased complexity of polypeptide synthesis. Such a function would reduce the apparent level of mistranslation. Edelmann and Gallant (1977) estimated a misreading probability per codon of 10^{-4} in *E. coli*, but the relative contributions of accurate translation and proteolysis are as yet unknown.

1.4 THE ENERGY REQUIREMENT FOR INTRACELLULAR PROTEIN DEGRADATION

An important feature of intracellular protein degradation is that it requires metabolic energy. This was first demonstrated by the discovery that protein degradation in liver slices was reduced by anaerobiosis or by the addition of cyanide or dinitrophenol (Simpson, 1953). Subsequently, a similar requirement for metabolic energy in *E. coli* (Mandelstam, 1958), chloroplasts (Liu and Jagendorf, 1984; Malek *et al.*, 1984) and mitochondria (Desautels and Goldberg, 1982a; Wheeldon and Lehninger, 1966) has been established. Moreover, in order to block proteolysis in *E. coli* the intracellular concentration of ATP must be severely reduced to 5-10% of that of growing cells (Olden and Goldberg, 1978).

An energy requirement for intracellular proteolysis was not anticipated on thermodynamic grounds (the hydrolysis of peptide bonds is exergonic) or from the behaviour of well characterized extracellular proteases. Thus the energy requirement may reflect mechanisms that confer specificity to the process and not an unusual pathway of peptide bond hydrolysis. Significant progress was made in understanding the energy requirement by the development of soluble, cell free systems from: rabbit reticulocytes (Etlinger and Goldberg, 1977), *E. coli* (Murakami *et al.*, 1979), and rat liver mitochondria (Desautels and Goldberg, 1982b). The properties of these systems are very similar:

1. They are soluble.
2. They have a neutral or slightly alkaline pH optimum, inactive below pH 5.0.
3. They degrade proteins to free amino acids.
4. They are not sensitive to inhibitors of lysosomal hydrolases (such as leupeptin or chloroquine).
5. They are sensitive to inhibitors of serine proteases and to chelating agents.

6. They require ATP and non-hydrolysable analogues of ATP do not support proteolysis.

Fractionation of these systems revealed the nature of the ATP dependence. Firstly, ATP-dependent proteases were isolated from all three (Swamy and Goldberg, 1981; Katayama-Fujimura *et al.*, 1987; Hwang *et al.*, 1987; Desautels and Goldberg, 1982b; Hough *et al.*, 1987; Waxman and Goldberg, 1987). In addition, a ubiquitin activating enzyme which is ATP-dependent was isolated from rabbit reticulocytes (Ciechanover *et al.*, 1981).

In the rabbit reticulocyte system ubiquitin, a 76 residue polypeptide (Schlesinger *et al.*, 1975; Ciechanover *et al.*, 1980; Wilkinson *et al.*, 1980), is covalently linked in an ATP-dependent reaction to a variety of protein substrates. One or more ubiquitin polypeptides are conjugated through their C-terminal glycine residues to a substrate protein via isopeptide bonds involving ε-amino groups of lysine residues (Hershko *et al.*, 1980; Goldknopf and Busch, 1977; Hunt and Dayhoff, 1977). Formation of an ubiquitin-protein conjugate may be the initial event in the degradation of proteins; many conjugates are rapidly digested in an ATP-dependent reaction (Hershko *et al.*, 1982; Chin *et al.*, 1982; Ciechanover *et al.*, 1984a). Hence in this system, modification by ubiquitination is thought to label a protein for degradation. However, this model will have to be reappraised in the light of the report that ubiquitin has intrinsic proteolytic activity (Fried *et al.*, 1987).

Studies on *E. coli* have failed to detect ubiquitin or ubiquitin-activating enzyme (Ciechanover *et al.*, 1984b) with one early exception (Goldstein *et al.*, 1975). In addition, despite the remarkable conservation seen in eukaryotes (Ciechanover *et al.*, 1984b) there has been no report of possible ubiquitin coding sequences in *E. coli*. However, it is possible that a different labelling reaction could serve a similar function in bacteria.

1.5 FACTORS CONFERRING SPECIFICITY TO INTRACELLULAR PROTEOLYSIS

Intracellular protein degradation in bacterial cells is a highly specific process. Individual protein species are digested at vastly different rates with half lives ranging from minutes (abnormal proteins and some regulatory proteins) to many hours (most cellular proteins). The origin of the specificity is unknown but one can envisage three possible sources:

1. Features of the proteins themselves may determine their rate of degradation.
2. The protease(s) involved may confer specificity.

3. An intermediary labelling process (comparable to ubiquitination) could confer specificity to protein degradation.

Although strong evidence to support any of these ideas is lacking, there is circumstantial evidence, drawn from many sources, which suggests that all three may be important in conferring specificity to the process.

1.5.1 Features of Proteins That Confer Specificity to Protein Degradation

Alterations in normal protein conformation, arising through mutation, incorporation of amino acid analogues, denaturation or premature chain termination, can markedly increase rates of degradation (see Goldberg and St. John, 1976), suggesting that features of proteins confer specificity to proteolysis. Furthermore, binding of substrates or co-factors to proteins has been found to influence rates of degradation (see Goldberg and Dice, 1974). Many studies, mainly carried out in eukaryotic systems, have revealed three categories of features which possibly determine rate of digestion: general properties, sequence-specific parameters and location (Rechsteiner *et al.*, 1987; see Table 1.1).

TABLE 1.1 Features of Proteins Proposed to Increase Rates of Degradation [a]

GENERAL PROPERTY	SEQUENCE-SPECIFIC PARAMETERS	LOCATION
Large size	PEST-sequence	Assembled/unassembled
Negative charge	RNase pentapeptide	Bound/diffusible
Hydrophobicity	α-amino terminus	
Thermal instability	His/Cys/Met oxidation	
High flexibility	Asn deamidation	
Proteolytic susceptibility		

a. Taken from Rechsteiner et al. (1987).

Early experiments using double isotope methods to measure relative rates of degradation of cellular proteins suggested that several general properties increased rates of degradation: large size (Dice *et al.*, 1973), negative charge (Dice and Goldberg, 1975a), and hydrophobicity (Segal *et al.*, 1976). However, exceptions to these general trends have been reported (Dice and Goldberg, 1975a; 1975b). Using other techniques,

thermal instability (McLendon and Radany, 1978), high flexibility (Perry *et al.*, 1979), and proteolytic susceptibility *in vitro* (Bond, 1971) have also been reported to increase rates of degradation.

The development of large scale micro-injection procedures has permitted direct examination of the correlation between these general properties and increased rates of degradation. Neff *et al.* (1981) injected a mixture of radiolabelled rat liver proteins into human fibroblasts and, in agreement with the proposed relationships, found that larger proteins were degraded faster than smaller ones, and negatively charged proteins were degraded faster than positively charged proteins. In contrast, Rogers and Rechsteiner (1985) using the same technique found that rates of degradation of 32 proteins of known primary and tertiary structure did not correlate with size, charge, content of hydrophobic residues, acylation of amino terminus, or the proportion of α-helical or β-sheet conformation, when injected into HeLa cells. Calorimetric data available for 22 of the 35 injected proteins revealed that thermal stability also did not correlate with degradative rates. However, both these studies involved only a small selection of cellular proteins and clearly, further studies are needed to clarify whether these general properties of a protein affect its rate of degradation.

Upon denaturation proteins become more susceptible to degradation by proteases *in vitro* (Linderstrom-Lang, 1952; Bennet, 1967; Rupley, 1967). This suggests that intracellular proteolytic pathways might preferentially digest proteins in transient, unfolded conformations. The correlation of thermal instability, high flexibility and proteolytic susceptibility of proteins with rapid degradation are possibly a reflection of higher rates of reversible unfolding (spontaneous denaturation) for some protein species. However, recent micro-injection studies have revealed that denaturation of a protein does not necessarily enhance its degradation *in vivo*. Katznelson and Kulka (1985) compared the degradation of native and denatured forms of serum albumin, β-lactoglobulin and cytochrome *c* in cultured rat hepatoma cells. Denatured β-lactoglobulin was digested three times faster than native β-lactoglobulin; the rate of degradation of cytochrome *c* did not alter following denaturation; and denatured serum albumin was degraded at a fifth of the rate of native form. Furthermore, Hough and Rechsteiner (1984) discovered that the Arrhenius activation energies for the degradation of various proteins micro-injected into HeLa cells were at least twice those of local protein unfolding, indicating that unfolding is not rate limiting in protein degradation *in vivo*. In another micro-injection study, Rote and Rechsteiner (1986) found that increased rates of hydrogen exchange, or breathing flexibility, which is

thought to reflect localized denaturation did not correlate with increased rates of protein degradation, although *in vitro* such a correlation held true with a range of proteases. In conclusion, it appears that unfolding of a protein does not necessarily increase its susceptibility to digestion *in vivo*.

Recently, several sequence-specific parameters of proteins have been proposed to correlate with increased rates of degradation:

1. Rogers *et al.* (1986) observed that the most rapidly degraded eukaryotic proteins of known sequence (10 proteins with half-lives less than 2 hours) had a feature in common: they contained one or more regions rich in proline (P), glutamate (E), serine (S) and threonine (T). These PEST regions, which vary from 12 to 60 residues in length, are generally flanked by clusters of positively charged amino acids. Inspection of 35 eukaryotic proteins which are slowly digested (half-lives between 20 and 220 hours) revealed that only 3 of them contained a PEST region. Further support for the hypothesis implicating PEST regions in susceptibility to proteolysis was given by subsequent surveys which showed that 23 out of 24 rapidly degraded eukaryotic proteins contained PEST regions (Rechsteiner *et al.*, 1987). However, the λ *N* and *c* II products, and the *E. coli* HtpR protein, which are rapidly digested *in vivo*, do not contain PEST regions (Rogers *et al.*, 1986).

2. Using a micro-injection procedure, Dice *et al.*, (1986) identified a pentapeptide sequence (Lys-Phe-Glu-Arg-Gln) of ribonuclease A that is required for the enhanced degradation of this protein in cultured human fibroblasts during serum deprivation. When a peptide containing the pentapeptide sequence was covalently attached at unspecified locations to ribonuclease S, lysozyme and insulin A chain, the conjugates were found to be degraded more rapidly under the same conditions (Backer and Dice, 1986).

3. Two experiments have suggested the importance of the α-amino terminus in determining rates of protein degradation. Firstly, Bachmair *et al.* (1986) reported that the specific amino acid at the α-amino terminus of a protein determines its rate of degradation (the "N-end" rule). Mutant β-galactosidases engineered to have different amino acids at their amino termini had different half-lives: Ser, Ala, Thr, Val and Gly conferred half-lives greater than 20 hours; Ile, Glu, Tyr and Gln, half-lives between 10 and 30 minutes; and Phe, Leu, Asp, Lys and Arg, half-lives less than 3 minutes. Wild type β-galactosidase has methionine at the amino terminus and has a half-life greater than 20 hours. In a different experiment, the importance of an unblocked amino-terminus for ubiquitin-dependent degradation

was demonstrated using reagents that differentially block α- and ε-amino groups (Hershko *et al.*, 1984).

4. Oxidation of histidine, cysteine and methionine *in vivo* may also be involved in protein degradation. Hershko *et al.* (1986) found that possession of an oxidized methionine increased the susceptibility of some proteins to ubiquitin-dependent degradation. Stadtman (1986) has proposed that oxidation of proteins by mixed-function oxidation systems leads to inactivation and also increases susceptibility to proteolysis. The identification of proteases in rat liver (Rivett, 1985) and *E. coli* (Roseman and Levine, 1987) which digest oxidized glutamine synthetase, but not the native enzyme, provide support for this proposal. Furthermore, prior to sporulation in *B. subtilis*, glutamine phosphoribosylpyrophosphate amido-transferase is first inactivated by oxidation and then rapidly degraded (Wong *et al.*, 1978).

5. Circumstantial evidence implicates deamidation of asparagine residues in controlling the rate of proteolysis. Robinson and Rudd (1974) demonstrated that in model peptides, rates of asparagine deamidation can be substantially affected by neighbouring residues. Changes in conformation following deamidation could determine the rates of degradation of proteins. Moreover, Clarke (1985) reported methylation of newly formed carboxyl groups at isoaspartate residues following asparagine deamidation, and this may label a protein for digestion.

The location of a protein may determine its rate of degradation. Dehlinger and Schimke (1970) first suggested that dissociation of multimeric enzymes would produce rapidly digested subunits. Similarly, the degradation rate of a protein might depend upon its association with macroscopic cellular structures, such as membranes or cytoplasmic filaments (Siekevitz, 1972). These suggestions have received considerable support from studies which demonstrated that ribosomal proteins (Abovich *et al.*, 1985), mitochondrial proteins (Reid and Schatz, 1982), globin (Shaeffer, 1983) and spectrin (Woods and Lazarides, 1985) are rapidly degraded unless assembled, and from the finding that neuron-specific creatine kinase and enolase, which associate with structural proteins during axonal transport, are remarkably stable (Brady and Lasek, 1981).

1.5.2 Occurrence of Highly Specific Proteases

Heterogeneity in the degradative rates of proteins in bacteria may reflect that a large number of highly specific proteases are involved in the process. However, this would seem to be an unsatisfactory method of conferring specificity because it is

metabolically expensive and requires a large coding capacity. Nevertheless, several highly specific proteases have been isolated, suggesting that they have an important role to play in conferring specificity.

The first highly specific protease to be purified from *E. coli* was RecA (Roberts *et al.*, 1978). Upon damage to DNA, RecA is activated and becomes a highly specific protease which cleaves the λ *c*I and *lexA* repressors (Roberts *et al.*, 1978; Little *et al.*, 1980) at a single Ala-Gly bond (Pabo *et al.*, 1979; Horri *et al.*, 1981). Recently, Little (1984) reported autodigestion of the λ *c*I and *lexA* repressors *in vitro* at the same Ala-Gly bond. This report and the failure to find other substrates question the possession of a highly specific proteolytic activity by RecA.

Many other intracellular proteases, including two ATP-dependent proteases, have been isolated from *E. coli* using non-specific assays (Pacaud and Uriel, 1971; Pacaud and Richaud, 1975; Cheng and Zipser, 1979; Strongin *et al.*, 1979; Swamy and Goldberg, 1981; Goldberg *et al.*, 1982; Pacaud, 1982; Katayama-Fujimura *et al.*, 1987; Hwang *et al.*, 1987). The properties of an ATP-dependent protease are described in Section 1.6. With one exception (protease Re, see below), little is known about the substrate specificity of these intracellular proteases *in vivo*.

Roseman and Levine (1987) purified a highly specific protease which degrades oxidized glutamine synthetase but not native enzyme. Oxidized glutamine synthetase is cleaved at an Ala-Gly peptide bond giving polypeptide products of molecular weights 4,500 and 47,000 (Roseman and Levine, 1987). Digestion of radiolabelled casein and insulin, but not serum albumin or haemoglobin, to acid-soluble fragments was observed (Roseman and Levine, 1987). Recently, this protease was shown to have similar properties to protease Re (Park *et al.*, 1988); an enzyme which had previously been identified by Goldberg *et al.* (1982).

Highly specific proteases have also been isolated from organisms other than *E. coli*. Setlow (1976; 1978) has purified two proteases from *B. megaterium* spores which have similar substrate specificity. Both cleave the small, basic proteins found in spores at a glutamate residue in regions which have similar pentapeptide sequences. They fail to degrade other protein, peptide, amide or ester substrates. A group of cysteine endopeptidases that require Ca^{2+} ions for activity (also called: calpains; calcium dependent proteinases, CDPs or CAPs; calcium activated neutral proteinases; CANPs; or calcium activated factors, CAFs) have recently been isolated from mammalian tissues (see Pontremoli and Melloni, 1986). Calpains do not appear to have general proteolytic activity, but provide limited cleavage of substrates necessary

for specific physiological responses. For example, human erythrocyte calpain rapidly degrades globin chains at single peptide bonds; between Lys-Ala in α and Lys-Ser in β chains, respectively (Melloni *et al.*, 1984). Many other extremely large, complex proteases (multicatalytic proteinases) have been isolated from mammalian tissues (see Bond and Butler, 1987). As yet, little is known about their substrate specificity *in vivo*. However, Rivett (1985) has described a protease which preferentially digests oxidized glutamine synthetase and ATP-dependent proteases which degrade ubiquitin-protein conjugates have been described (Hough *et al.*, 1987; Waxman *et al.*, 1987; Fagan *et al.*, 1987). In addition, Katunuma *et al.* (1971a; 1971b) have identified proteases specific for pyridoxal or NAD requiring enzymes in several mammalian tissues.

1.5.3 Post-Translational Modification Reactions Confer Specificity to Protein Degradation

The idea that post-translational modification reactions (also called labelling or marking reactions) could confer specificity to protein degradation arises from the discovery that modified proteins are more susceptible to digestion than unmodified proteins. Four such modification reactions have been identified:

1. The phosphorylation of serine or threonine residues (Toyo-Oka, 1982; Bergstrom *et al.*, 1978; Hall *et al.*, 1979; Parker *et al.*, 1984).
2. The formation of mixed disulphide derivatives of cysteine residues (Offermann *et al.*, 1984).
3. The conjugation of ubiquitin with ε-amino groups of lysine residues (Hershko *et al.*, 1982).
4. The oxidation of amino acid residues by mixed-function oxidation systems (Levine *et al.*, 1981).

Thus it seems that in some cases protein degradation may occur in a two step process: firstly, the protein is modified; secondly, the modified protein is digested. Possibly, the heterogeneity in rates of degradation reflects that enzymes with different substrate specificities are involved in the modification reaction.

The most fully characterized modification reaction in bacteria is oxidation by mixed-function oxidation systems (Levine, 1983a; 1983b). The first step in the degradation of *E. coli* glutamine synthetase is oxidation of one of sixteen histidine residues (Levine, 1983a). This occurs under conditions in which glutamine synthetase is not required by the cell: substrates are not available, or glutamine is present (Levine, 1983b). The modified enzyme, which is catalytically inactive, is susceptible to

proteolysis (Levine *et al.*, 1981; Farber and Levine, 1982). Ten out of twenty-five metabolic enzymes are inactivated by mixed-function oxidation systems suggesting that oxidative modification may be generally involved in protein degradation (Fucci *et al.*, 1983). If this is the case, then protein degradation will be influenced by factors which affect the activity of mixed-function oxidation systems, such as: the partial pressure of oxygen and the concentrations of iron and copper ions; the availability of alternative electron donors and acceptors of the flavin components of the mixed-function oxidation system; cytochrome P-450 and non-haem iron sulphur proteins; scavengers of activated oxygen species (vitamin E, ascorbate, superoxide dismutase, catalase, peroxidase, Mn^{2+}); and the concentrations of physiological metal chelators (Stadtman, 1986).

The most direct evidence that modification reactions could confer specificity to proteolysis comes from studies on the formation of ubiquitinated proteins in rabbit reticulocytes. Three enzymic activities are thought to be involved in this process: ubiquitin-activating enzyme, E1 (Ciechanover *et al.*, 1981; Ciechanover *et al.*, 1982); ubiquitin carrier protein, E2 (Hershko *et al.*, 1983); and E3 (Hershko *et al.*, 1983; Hershko *et al.*, 1986). Five species of E2, which differ in their substrate specificities, have been identified (Pickart and Rose, 1984). Four E2s could catalyse the transfer of ubiquitin to small amines in the absence of E3 (Pickart and Rose, 1984). Two E2s could catalyse the transfer of ubiquitin to small basic proteins (histones and cytochrome *c*) in an E3-independent reaction (Pickart and Rose, 1984). Only a single E2 was found to function in E3-dependent ubiquitin conjugation with creatine phosphokinase, reduced/carboxy-methylated serum albumin and oxidized RNase, and in E3-dependent degradation of serum albumin (Pickart and Rose, 1984). Thus, regulation of protein degradation could occur via controls acting on specific E2s.

1.5.4 Current Status

The study of factors conferring specificity to intracellular protein degradation in prokaryotes and eukaryotes is at a stage where there is, at present, no accepted background of established facts. Different groups using separate approaches believe that they have identified important factors, but no synthesis has been made, and it is unlikely that all of the present theories can be reconciled. The number of publications in the field in the last few years has been large and it is difficult to know which 'facts' from each group have become obsolete and which are still believed to be true. Investigation into the properties of the proteases involved is a promising approach. A novel group of proteases which are dependent upon ATP have been identified. Their

role in conferring specificity to intracellular proteolysis in bacteria is currently undergoing examination.

1.6 THE ROLE OF ATP-DEPENDENT PROTEASES IN INTRACELLULAR PROTEIN DEGRADATION IN *E. coli*

Genetical and biochemical studies of protein degradation in *E. coli* have revealed the importance of the ATP-dependent protease La in conferring specificity to the process. The recent discovery of another ATP-dependent protease in *E. coli* (Katayama-Fujimura *et al.*, 1987; Hwang *et al.*, 1987) gives further support to the following model for protein degradation:

1. The first step is limited cleavage by a highly specific ATP-dependent protease.
2. The second step involves rapid digestion of the protein fragments by non-specific proteases and peptidases to amino acids.

In this model the specificity and ATP dependence of protein degradation arise from properties of the proteases involved in the step 1, which can be regarded as a form of proteolytic modification. Evidence for the model is described below.

1.6.1 Genetical Studies on Protein Degradation in *E. coli*

Bukhari and Zipser (1973) first isolated mutants of *E. coli* (*deg*) which had a defect in the degradation of truncated polypeptides of β-galactosidase. They selected for these mutants by using strains which could not grow unless nonsense mutants of β-galactosidase were stabilized to allow interallelic complementation. These *deg* mutants were found to be indistinguishable by any of their phenotypes or mapping characteristics to previously described *lon* mutants (Shineberg and Zipser, 1973; Gottesman and Zipser, 1978). Mutations in the *lon* locus of *E. coli* result in many phenotypic changes:

1. Over-production of capsular polysaccharide resulting in mucoid colonies (Markovitz, 1964).
2. Transcription derepression of the *gal* operon (Hua and Markovitz, 1972; 1974; Mackie and Wilson, 1972).
3. Enhanced sensitivity to U.V. light or radiomimetic agents, with concomitant filamentation (Howard-Flanders *et al.*, 1964).
4. Defective establishment and maintenance of certain plasmids (Takano, 1971; Falkinham, 1979).

5. Defective lysogeny of bacteriophage P1 (Takano, 1971) and bacteriophage λ (Walker *et al.*, 1973).
6. Deficient degradation of abnormal polypeptides (Bukhari and Zipser, 1973; Gottesman and Zipser, 1978; Simon *et al.*, 1979; Young and Davis, 1983), and several normal proteins (λ *N* product, Gottesman *et al.*, 1981; SulA, Mizusawa and Gottesman, 1983; RcsA, Torres-Cabassa and Gottesman, 1987); but no deficiencies in the degradation of normal, stable proteins elicited by starvation (Goldberg and St. John, 1976; Maurizi *et al.*, 1985) or several short-lived, normal proteins (λ *O, xis*, and *c*II products, Gottesman *et al.*, 1981; UmuD and C, Marsh and Walker, 1985; HtpR, Grossman *et al.*, 1985).

Cloning of the *lon* gene (Zehnbauer and Markovitz, 1980; Schoemaker and Markovitz, 1981) permitted the identification and purification of a polypeptide of molecular weight 94,000 as the gene product (Schoemaker and Markovitz, 1981; Zehnbauer *et al.*, 1981), which has the following properties:

1. A proteolytic activity which is coupled to a protein-stimulated ATPase activity (Charette *et al.*, 1981).
2. A non-specific nucleic acid binding activity (Zehnbauer *et al.*, 1981).
3. A DNA-stimulated ATPase activity (Charette *et al.*, 1984).
4. A tetrameric structure of molecular weight about 350,000 (Charette *et al.*, 1982).

Insertional mutagenesis revealed that the *lon* gene is not essential for growth of *E. coli* (Maurizi *et al.*, 1985). The *lon* gene product has been shown to belong to a unique group of proteins that are heat-shock inducible (Goff *et al.*, 1984; Phillips *et al.*, 1984). The nucleotide sequence of the *lon* gene has revealed that the gene product has a lower predicted molecular weight than previous estimates (87,000 not 94,000; Chin *et al.*, 1988). Computer-assisted comparisons revealed only that the *lon* gene product contains a sequence similar to one found in nucleotide binding proteins (Chin *et al.*, 1988).

Chung and Goldberg (1981) demonstrated that the *lon* gene product is identical to the ATP-dependent protease La. The discovery that *lon* mutants are defective in the degradation of some proteins and not others, suggests that protease La is highly specific.

TABLE 1.2 Proteases of *E. coli* [a]

ENZYME	SUBSTRATES [b,c]	M_r (x 10^{-3})	INHIBITORS [c]
Soluble endoproteases			
Do	globin, casein	540	D
Re [d]	globin, casein	82	D, E, O, TPCK
Mi	globin, casein	110	D, E, O
Fa	globin, casein	110	D, E, O, TPCK
So	globin, casein	140	D, TPCK
La (requires ATP)	globin, casein	450	D, E, NEM
Ci	insulin	125	E, O
Pi (Protease III)	insulin, auto-α [e]	110	E, O, PHMB
Protease I	NAPNE	-	D
Protease III	BAEE	-	D, TLCK
ISP-L-Eco	Z-Ala-Ala-Leu PNA	55	D, E
Membrane-associated endoproteases			
Protease IV	Z-Val ONP	34	D
Protease V	Z-Phe ONP	-	D

a. Taken from Miller (1987).

b. Substrates used to assay each enzyme are given.

c. Abbreviations: D, diisopropylfluorophosphate; E, EDTA; O, o-phenanthroline; TPCK, N-tosyl-phenylalanine chloromethyl ketone; NEM, N-ethyl maleimide; PHMB, p-hydroxymercuribenzoate; NAPNE, N-acetyl-phenylalanine β-naphthyl ester; BAEE, N-benzoylarginine ethyl ester; TLCK, N-tosyllysine chloromethyl ketone; Z, benzyloxycarbonyl; PNA, p-nitroanilide; ONP, p-nitrophenyl ester.

d. Protease Re has also been isolated as an enzyme that selectively degrades oxidized glutamine synthetase (Park et al., 1988).

e. Auto-α is a small fragment of β-galactosidase.

1.6.2 Biochemical Studies on Protein Degradation in *E. coli*

Murakami *et al.* (1979) first produced a soluble extract of *E. coli* which retained the important features of intracellular protein degradation: the extract selectively degraded abnormal polypeptides, and showed energy (ATP)-dependent proteolysis. Fractionation of such an extract led to the identification of eight soluble endoproteases, one of which (protease La) was ATP-dependent (Swamy and Goldberg, 1981; Goldberg *et al.*, 1982). The properties of these proteases and of other proteases independently purified from *E. coli* are shown in Table 1.2. Many peptidases (see

Miller, 1987) have also been isolated from *E. coli*. Originally, only one ATP-dependent protease (La) was isolated (Swamy and Goldberg, 1981), but recently another has been identified (Katayama-Fujimura *et al.*, 1987; Hwang *et al.*, 1987). Surprisingly, the genes encoding only two of the proteases described in Table 1.2 have been identified: the *lon* gene encodes protease La (Charette *et al.*, 1981; Chung and Goldberg, 1981); and the *ptr* gene encodes protease III (Cheng *et al.*, 1979).

The discovery that the degradation of both abnormal and normal proteins in *E. coli* is drastically reduced when the generation of ATP is prevented (see Goldberg and St. John, 1976) reveals the importance of ATP-dependent proteases. Furthermore, *lon* mutants are defective in the same initial endoproteolytic cleavage of a truncated β-galactosidase polypeptide, as that blocked by ATP depletion (Kowit and Goldberg, 1977). It appears, therefore, that ATP-dependent proteases may catalyse the initial steps in the degradation of proteins.

Protease La was the first member of a novel group of ATP hydrolysis-dependent proteases to be isolated. Other members of this group include the multi-component, ATP-dependent protease recently isolated from *E. coli* (Katayama-Fujimura *et al.*, 1987; Hwang *et al.*, 1987), the ATP-dependent protease isolated from mitochondria (Desautels and Goldberg, 1982b), and the ATP/ubiquitin-dependent proteases isolated from several mammalian tissues (Hough *et al.*, 1987; Waxman *et al.*, 1987; Fagan *et al.*, 1987). In order to gain an understanding of the mechanism of peptide bond cleavage involving ATP hydrolysis, the properties of the ATP-dependent protease La have been studied and are listed below:

1. Protease La degrades [^{14}C]-globin and [^{3}H]-casein to acid-soluble fragments in the presence of ATP, but not non-hydrolysable analogues of ATP (Larimore *et al.*, 1982; Charette *et al.*, 1981).
2. Protease La has a protein substrate-stimulated ATPase activity (Charette *et al.*, 1981; Waxman and Goldberg, 1982).
3. DNA stimulates the ATP-dependent proteolytic activity of protease La (Chung and Goldberg, 1982).
4. The proteolytic and ATPase activities of protease La are tightly coupled; about two ATP molecules are hydrolysed for each peptide bond cleaved (Waxman and Goldberg, 1982; Menon *et al.*, 1987).
5. Neither protease La nor its protein substrate is adenylated or phosphorylated

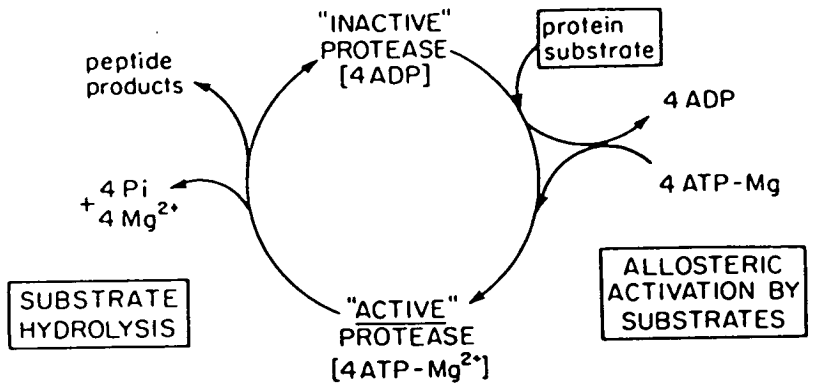
during ATP-dependent proteolysis (Larimore *et al.*, 1982; Waxman and Goldberg, 1985; Goldberg and Waxman, 1985).

6. Non-hydrolysable analogues of ATP can support the cleavage of small fluorogenic peptides, but not proteins, by protease La (Goldberg and Waxman, 1985).
7. Protease La has two recognition sites for protein substrates: the active (catalytic) site and the regulatory (allosteric) site (Waxman and Goldberg, 1986).
8. Occupancy of the regulatory site by a protein substrate stimulates the proteolytic and ATPase activities of protease La (Waxman and Goldberg, 1986). Stimulation of the proteolytic activity occurs by promoting the release of ADP and the binding of ATP (Menon and Goldberg, 1987b).
9. Protease La has two high affinity sites for ATP, and two sites showing lower affinities (Menon and Goldberg, 1987a).
10. Protease La has a higher affinity for ADP than ATP (Menon and Goldberg, 1987a)
11. ADP is a potent inhibitor of the proteolytic activity of protease La (Waxman and Goldberg, 1982; 1985).

The model shown in Figure 1.1 of the relationship between ATP binding and hydrolysis in the mechanism of action of protease La has been proposed by Menon and Goldberg (1987b). The sequence of events is:

1. Interaction of a protein substrate with inactive protease La at the regulatory site leads to release of bound ADP molecules. Concomitantly, ATP-Mg species become associated with the enzyme and bring about conversion to its active form.
2. Active protease La cleaves peptide bonds and in doing so two molecules of ATP are hydrolysed per bond cleaved.
3. The phosphate groups and Mg^{2+} ions are released, as are the ADP moieties, provided that the regulatory site remains occupied.
4. Binding of ATP-Mg species allows the cycle to continue.
5. Once the protein is completely hydrolysed, protease La returns to an inactive state in which ADP is tightly bound. It remains in this state until another protein substrate interacts with the enzyme, and so degradation of essential cell proteins is prevented.

FIGURE 1.1 Schematic Model of the Mechanism of Action of Protease La



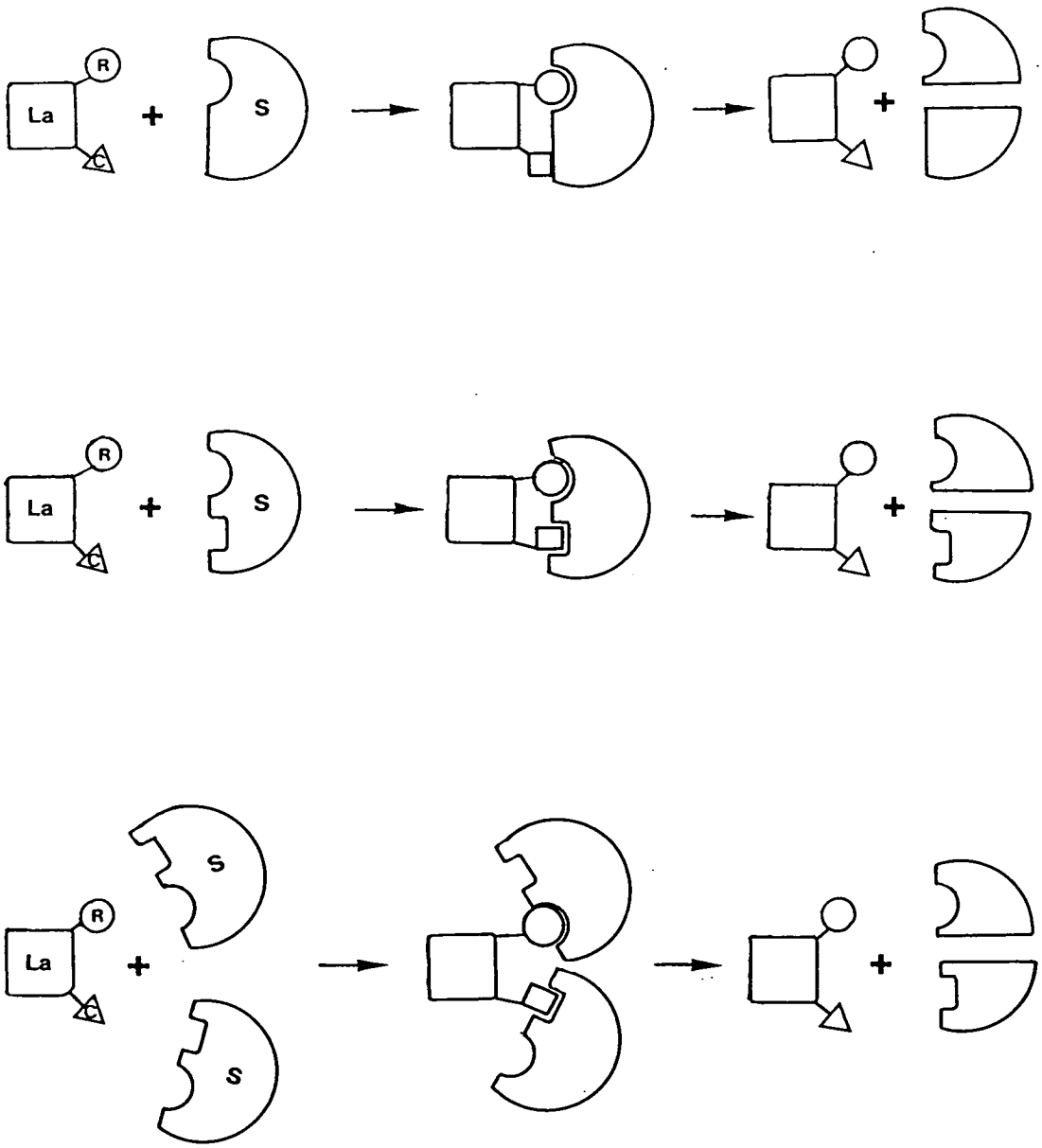
Taken from Menon and Goldberg (1987b).

This model begins to explain how extensive proteolysis of essential proteins by protease La is prevented *in vivo*, but fails to provide insight into three important, related questions concerning the interaction of proteins with protease La:

1. Do the regulatory and catalytic sites of protease La interact with the same protein molecule or with different protein molecules?
2. Does the specificity shown by the enzyme reside in the regulatory site or the catalytic site, or both?
3. What are the features of proteins that enable them to interact with protease La and hence lead to their cleavage?

Until these questions are answered, one can envisage three possible models for the interaction of proteins with protease La (Figure 1.2). In model A, a protein interacts with the regulatory site (converting the enzyme to the active form) and simultaneously with the catalytic site. Features of the protein recognized by the regulatory site confer specificity in this model, and although the catalytic site shows no specificity the geometry of the interaction limits the number of peptide bonds which can be cleaved. Model B describes the hypothesis that features of the protein are recognized by both the regulatory and catalytic sites conferring a high degree of specificity. Model C is similar to model B except that two protein molecules must

FIGURE 1.2 Schematic Models of the Interaction of Proteins With Protease La



S: a substrate protein; R: a regulatory site; C: a catalytic site.

interact with protease La to allow peptide bond cleavage. Initially, one molecule interacts with the regulatory site converting the enzyme to the active form. The catalytic site of the active enzyme only interacts with substrates, which are then cleaved.

In conclusion, since the identification of the ATP-dependent protease La much has been discovered about the regulation of its activity *in vivo*. However, a great deal remains to be learned about the features of proteins which determine their susceptibility to cleavage by protease La.

1.7 Aim of This Project

Intracellular protein degradation in bacterial cells is a highly specific process. At present, the features of this process which confer specificity are unknown. However, information from genetical and biochemical studies on *E. coli* suggests that ATP-dependent proteases play a key role in protein degradation. They appear to be highly selective endopeptidases which may be involved in the early steps of the digestion of a protein to amino acids. The aim of this project has been to investigate this possibility by studying protease La *in vitro* to address the following questions:

1. Does protease La digest native proteins?
2. How many peptide bonds in a substrate are cleaved by protease La?
3. What degree of selectivity is shown by protease La?
4. What are the features of substrates recognized by protease La?

This type of study may clarify whether the ATP-dependent proteolytic activity of protease La has a significant role in conferring specificity to proteolysis in bacterial cells. Moreover, knowledge of the features of proteins that confer sensitivity to protease La may reveal the basis of the recognition process. Indeed, if similar studies are carried out on other ATP-dependent proteases it may eventually become possible to predict susceptibility to intracellular proteolysis. A better understanding of the fundamental process of intracellular proteolysis may contribute towards a clearer perception of other important biological processes such as gene regulation and senescence, and it may also have implications for the improvement of expression systems in biotechnology. Clearly, there is great incentive for investigation into the action of ATP-dependent proteases in bacteria.

CHAPTER 2

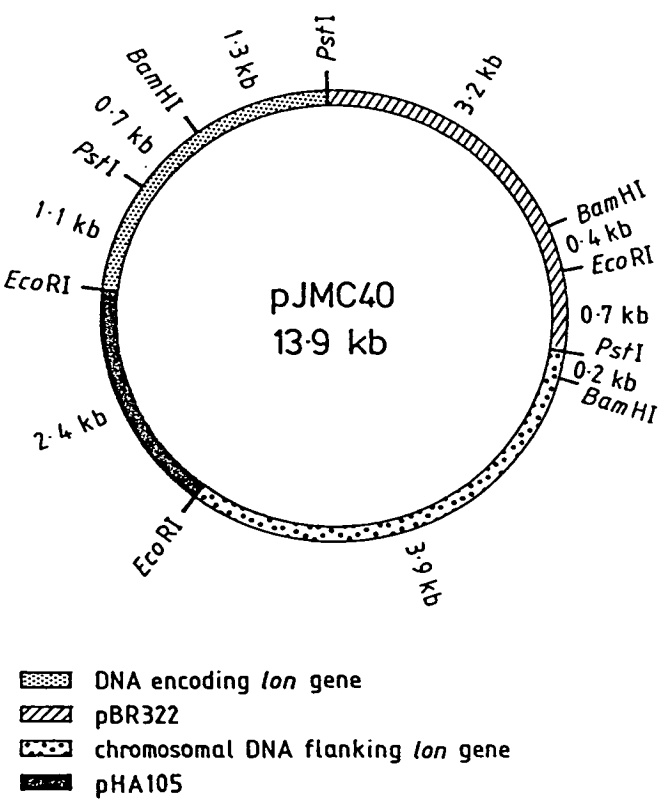
Results

2.1 PURIFICATION OF THE *E. coli* ATP-DEPENDENT PROTEASE La

2.1.1 An Over-Expressing Strain for the Purification of Protease La

The ATP-dependent protease La is encoded by the *lon* gene (Chung and Goldberg, 1981). Using plasmids pSC101 and pHA105 as vectors, Zehnbauer and Markovitz (1980) cloned a 12.5kb fragment of DNA, containing the *lon* gene, from a F' episome. During studies to identify the product of the *lon* gene, Schoemaker and Markovitz (1981) subcloned the 12.5kb fragment of DNA encoding the *lon* gene in the high copy number plasmid pBR322 to give plasmid pJMC40 (Figure 2.1).

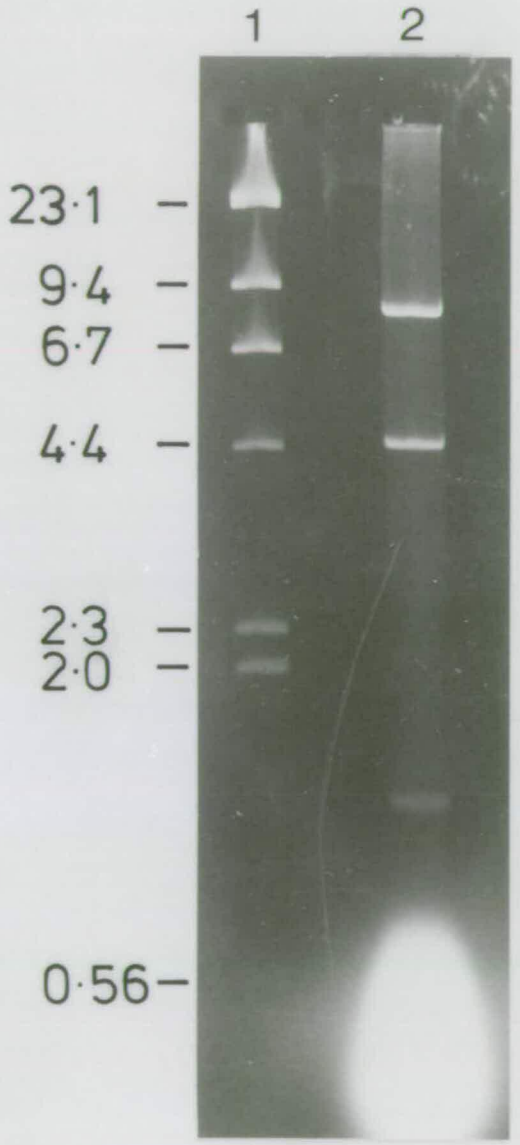
FIGURE 2.1 Restriction Map of pJMC40



Taken from Schoemaker and Markovitz (1981).

Dr. A. Markovitz provided sufficient pJMC40 to transform *E. coli*, creating a Tc^R strain which over-produces protease La (Chung and Goldberg, 1981). However,

FIGURE 2.2 Digestion of pJMC40 with *Bam*HI



DNA fragments were separated by electrophoresis through a 0.7% agarose gel, and detected under U.V. light after staining with ethidium bromide.

Lane 1: λcI857 DNA digested with HindIII giving DNA fragments of 23.1, 9.4, 6.7, 4.4, 2.3, 2.0, and 0.56kb.

Lane 2: pJMC40 digested with BamHI giving DNA fragments of 7.9, 4.8, and 1.2kb.

plasmids containing the *lon* gene, within strains over-producing protease La, have been found to be unstable (Goff and Goldberg, 1987), probably because of deleterious effects of high levels of protease La. Consequently, to verify Tc^R transformants contained pJMC40, plasmid was isolated and digested with *Bam*HI (Figure 2.2). The sizes of the DNA fragments obtained (7.9kb, 4.8kb and 1.2kb) are in good agreement with those predicted from the restriction map of pJMC40 (Figure 2.1). *E. coli*/pJMC40 was stored in glycerol at -20°C but because pJMC40 may be unstable in transformed cells a large scale preparation of pJMC40 was also carried out.

For purification of protease La, large quantities of *E. coli*/pJMC40 were grown and stored at -20°C. To confirm the cells contained pJMC40 before use, plasmid was isolated and digested with *Bam*HI giving DNA fragments of sizes characteristic of pJMC40.

2.1.2 Radiolabelling of Proteins *In Vitro* by Reductive Methylation

A convenient assay used in the purification of proteases relies upon the digestion of protein substrates to peptides soluble in 10% TCA. The sensitivity of this assay can be greatly increased by using radiolabelled proteins, which has allowed the detection (Swamy and Goldberg, 1981) and partial purification of protease La (Larimore *et. al.*, 1982).

The method of Rice and Means (1971) was used to radiolabel casein: free amino groups are converted to [^{14}C]-methyl and [^{14}C]-dimethyl derivatives by reaction with [^{14}C]-formaldehyde and the powerful reducing agent sodium borohydride. In comparison with Swamy and Goldberg (1981), a very low incorporation of label was achieved: about 0.5 to 1% of label was incorporated to give a specific activity of 5-9 x 10^5 cpm mg^{-1} casein. Unfortunately, ATP-stimulated proteolytic activity was not detected in extracts of *E. coli*/pJMC40 with this low specific activity [*methyl*-^{14}C]-casein (for clarity [*methyl*-^{14}C] shall be referred to as [^{14}C]). Consequently, an improved method of radiolabelling proteins was sought.

Substitution of sodium borohydride by the weaker reducing agent sodium cyanoborohydride, which can reduce Schiff bases but not aldehydes and ketones at neutral pH, increases labelling efficiency two to four times and eliminates side reactions caused by sodium borohydride (Jentoft and Dearborn, 1979). Furthermore, the reaction conditions necessary for efficient labelling are not as stringent when using sodium cyanoborohydride compared to sodium borohydride (Jentoft and Dearborn, 1979). When a six-fold excess of formaldehyde to free amino groups is

used, about 90% of free amino groups are converted to dimethyl derivatives; whereas equimolar amounts of formaldehyde and free amino groups leads to about 70% of the formaldehyde being covalently bound (Jentoft and Dearborn, 1979).

A difference in susceptibility of radiolabelled proteins to hydrolysis by protease La was reported by Chung and Goldberg (1981). Consequently, a range of proteins were radiolabelled to high specific activity (Table 2.1) by the method of Jentoft and Dearborn (1979) using a two-fold excess of [^{14}C]-formaldehyde to free amino groups. The incorporation of radiolabel into proteins using this method (Table 2.1) was very much greater than the 0.5 to 1% incorporation achieved by the method of Rice and Means (1971). A possible explanation for the difference could be the inability of sodium cyanoborohydride to reduce [^{14}C]-formaldehyde directly to [^{14}C]-methanol under the conditions used. The reason for the variability of the amount of label incorporated into different proteins was not understood but was, perhaps, related to the accessibility of free amino groups.

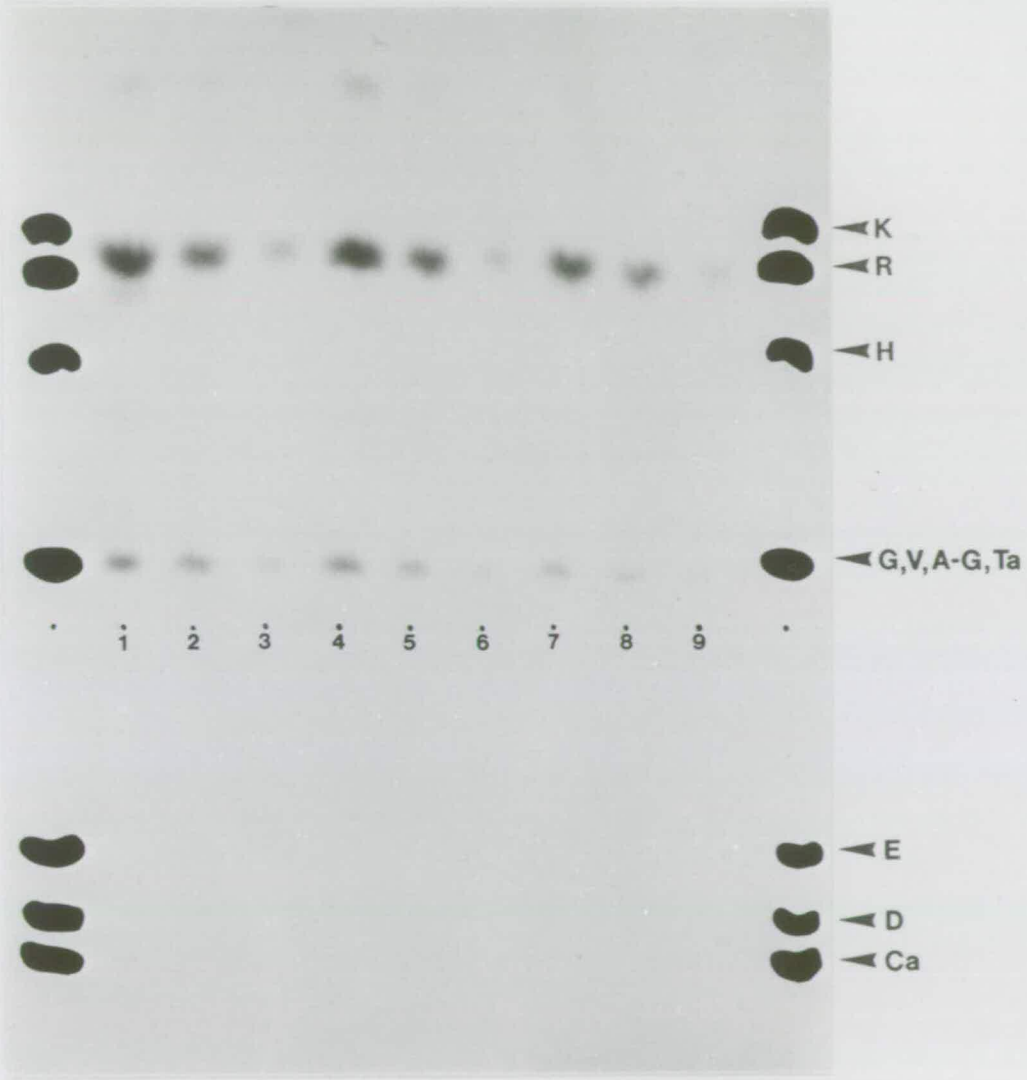
TABLE 2.1 Specific Activity of Proteins Radiolabelled by the Method of Jentoft and Dearborn (1979)

PROTEIN	SPECIFIC ACTIVITY (cpm mg^{-1})	PERCENTAGE INCORPORATION
β-lactoglobulin	1.4×10^7	38
casein	1.3×10^7	50
β-lactamase	7.4×10^6	13

Using [^{13}C]-NMR spectroscopy of proteins labelled by reductive methylation under optimal conditions, Jentoft and Dearborn (1979) showed: the only groups labelled were the ε-amino groups of lysine residues and the amino terminus; intermolecular and intramolecular cross-linking did not occur. The specificity of reductive methylation of proteins under the radiolabelling conditions employed was examined by amino acid analysis (Figure 2.3) and SDS-PAGE (Figure 2.4). After acid hydrolysis the products of the radiolabelling process resolved by HVPE at pH 6.5 were: a strongly basic species, probably [^{14}C]-methyl-lysine, which was the major product; neutral species which could be derivatives of nucleophilic amino acids or, in the case of β-lactoglobulin, [^{14}C]-methyl-leucine since a leucine residue is N-terminal (the

FIGURE 2.3 Amino Acid Analysis of [^{14}C]-Proteins

1 HVPE at pH 6.5



Various amounts (100,000, 50,000, and 20,000cpm) of [^{14}C]-proteins (0.7–1.8 x 10^7cpm mg^{-1}) were hydrolysed with acid and the products resolved by HVPE at pH 6.5. The paper was stained and also exposed to X-ray film to produce an autoradiograph. The positions of the amino acid standards are indicated. Ta: taurine.

Lanes 1–3: β-lactamase.

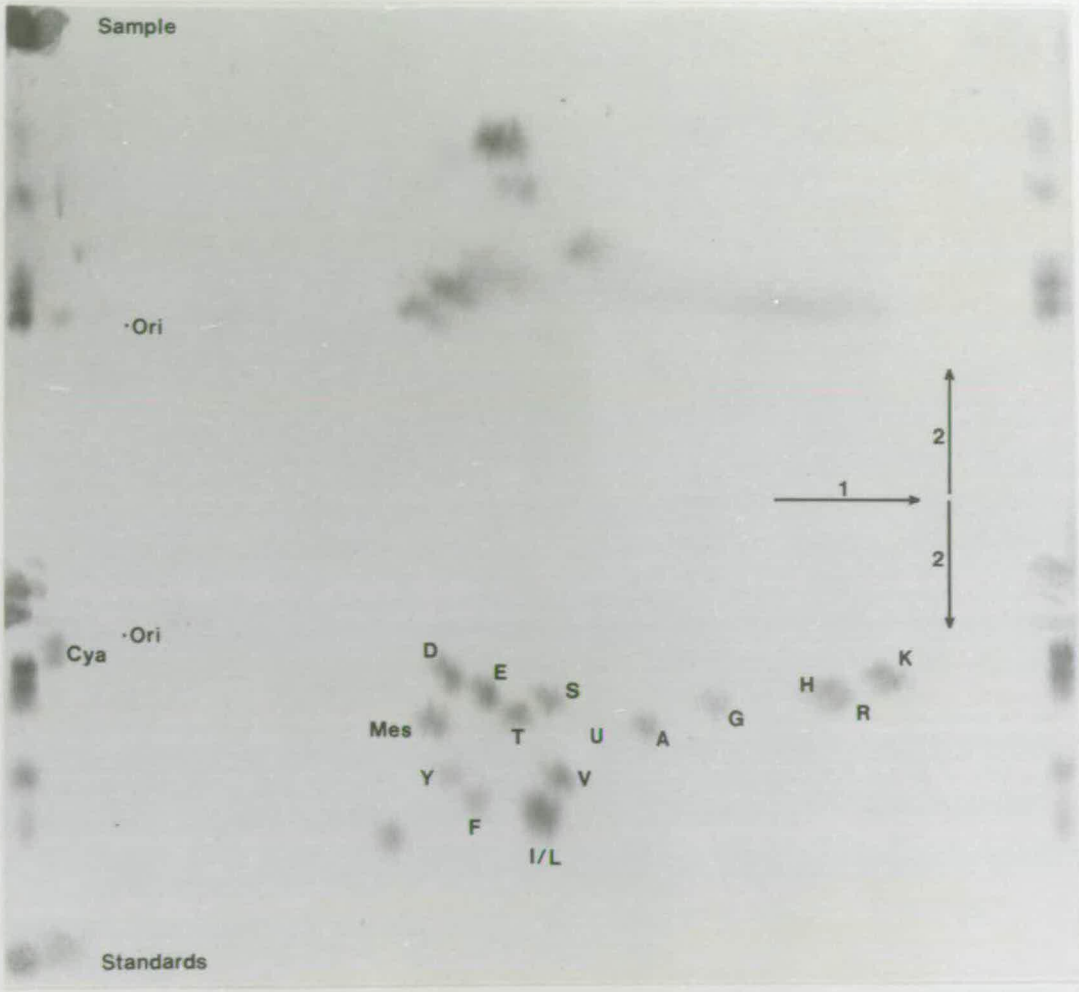
Lanes 4–6: β-lactoglobulin.

Lanes 7–9: casein.

FIGURE 2.3 (continued)

2 Two-Dimensional Separation

A

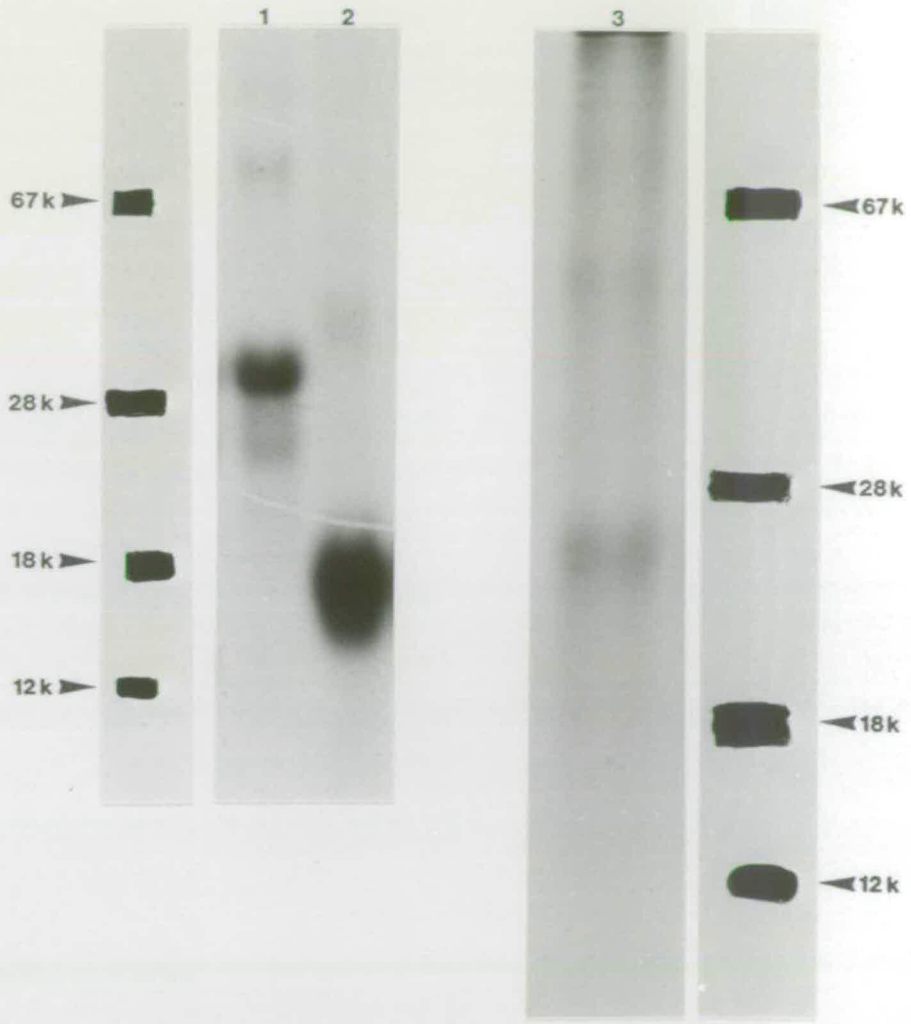


B

[^{14}C]-β-lactoglobulin (50μg, 1.0 x 10^7cpm mg^{-1}) was hydrolysed with acid and the products resolved in the first dimension by HVPE at pH 2.0 (1), and then in the second dimension by descending chromatography (2). The paper was stained and also exposed to X-ray film to produce an autoradiograph. The positions of the amino acid standards separated under identical conditions are indicated. Ori: origin; Cya: cysteic acid; Mes: methionine sulphone.

A stained paper; B autoradiograph.

FIGURE 2.4 SDS-PAGE of [^{14}C]-Proteins



10µg of each [^{14}C]-protein preparation (0.7–1.4 x 10^7cpm mg^{-1}) was resolved by SDS-PAGE (Anderson et al., 1983). The gel was stained (Steck et al., 1980) and exposed to X-ray film to produce an autoradiograph. The positions of molecular weight markers (BSA, 67,000; β-lactamase, 28,000; β-lactoglobulin, 18,000; cytochrome c, 12,000) are indicated.

Lane 1: β-lactamase.

Lane 2: β-lactoglobulin.

Lane 3: casein.

N-terminal amino acid of α-casein is arginine and of β-lactamase, lysine); and an extremely basic species of unknown origin (Figure 2.3.1). To characterize these radiolabelled species further, a two-dimensional separation of an acid hydrolysate of [^{14}C]-β-lactoglobulin was performed (Figure 2.3.2). The major species detected was probably [^{14}C]-methyl-lysine since it migrated to a position corresponding to basic amino acids. Several minor species were also detected but they could not be identified. Intermolecular cross-linking may have occurred to a limited extent since products corresponding to twice the molecular weight of the radiolabelled proteins were detected by SDS-PAGE (Figure 2.4). However these, and other radiolabelled products, may have been contaminants of the protein preparations (Figure 2.4). In conclusion, reductive methylation under these experimental conditions leads to modification of residues other than lysine. The nature of these modifications are not understood but they probably include intermolecular cross-linking.

TABLE 2.2 ATP-Stimulated Proteolytic Activity in an Extract of *E. coli*/pJMC40

[^{14}C]-PROTEIN	PERCENTAGE RADIOLABEL ACID-SOLUBLE [a]			
	+ CELL EXTRACT		- CELL EXTRACT	
	+ ATP	- ATP	+ ATP	- ATP
casein	10.9, 12.2	8.98, 8.45	2.16	2.31
β-lactoglobulin	22.5, 23.1	9.11, 8.10	1.91	2.00
β-lactamase	9.09, 8.63	7.44, 7.58	5.68	5.78

a. Assays contained 25mM Tris/HCl pH 8.0, 15mM $MgCl_2$, and 5-30μg [^{14}C]-protein (1.2-1.4 x 10^7cpm mg^{-1}) in a final volume of 500μl. ATP (final concentration 3mM) and 200μl cell extract were added as indicated. After incubation at 37°C for 80min, acid-soluble radioactivity was determined.

To determine if assays using radiolabelled proteins of high specific activity could detect ATP-stimulated proteolytic activity in *E. coli*/pJMC40, an extract was prepared, using the method of Swamy and Goldberg (1981), and assays carried out (Table 2.2). Although proteolytic activity was greater in the presence of 3mM ATP with all of the radiolabelled proteins, the greatest increase occurred when [^{14}C]-β-lactoglobulin was used as a substrate. The variability of the increase in proteolytic activity upon addition of 3mM ATP with different radiolabelled proteins may reflect the specificity of protease La or other ATP-dependent proteases; or

alternatively, may reflect differences in solubility of the peptide products in 10% TCA (or a combination of these). Whatever the reason, [^{14}C]-β-lactoglobulin, labelled to high specific activity (1-2 x 10^7 cpm mg^{-1}) by the method of Jentoft and Dearborn (1979), was used as a substrate in subsequent assays during the purification.

2.1.3 Chromatography Used in Purification of Protease La

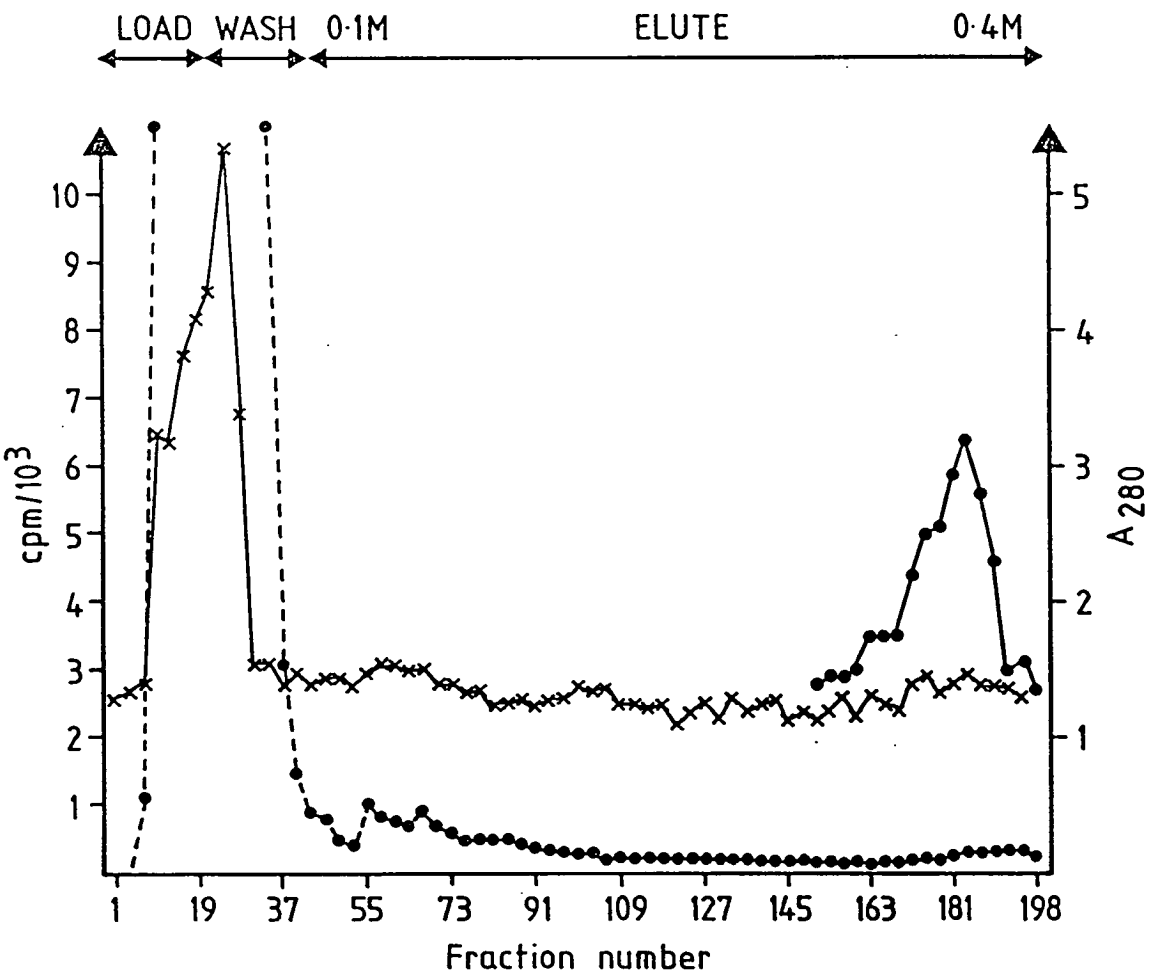
An extract of *E. coli*/pJMC40, displaying proteolytic activity, was fractionated by phosphocellulose (Figure 2.5) and DEAE-cellulose (Figure 2.6) chromatography as described by Zehnbauer *et al.* (1981). Further purification of protease La was achieved by gel filtration (Figure 2.7) as described by Goldberg *et al.* (1982). The concentration and purity of active fractions after gel filtration was assessed by SDS-PAGE (Figure 2.8). The most concentrated fractions were pooled and stored at -70°C after addition of EDTA and DTT. Protease La was 90% pure as judged by densitometry (using a Shimadzu CS-930) of a Coomassie Blue stained polyacrylamide gel (Figure 2.9).

2.1.4 Proteolytic Activity of the Protease La Preparation

To determine if the protease La preparation was contaminated by proteases, it was assayed with a range of radiolabelled proteins, in the presence and absence of ATP (Table 2.3). With the exception of [^{14}C]-β-lactamase, which was degraded very poorly, insignificant amounts of the radiolabelled proteins were rendered acid-soluble when incubated with protease La in the absence of ATP; but in the presence of ATP, significant amounts were rendered acid-soluble. Increasing the amount of the protease La preparation used in assays led to greater recovery of acid-soluble material in the presence of ATP; whereas in the absence of ATP, even with the largest amount of the preparation, insignificant recovery of acid-soluble material was attained (Table 2.3). Thus, at the detection limit of these assays, the protease La preparation was found to be free from contaminating proteases. However, these assays would have failed to detect contamination by highly specific proteases or by other ATP-dependent proteases.

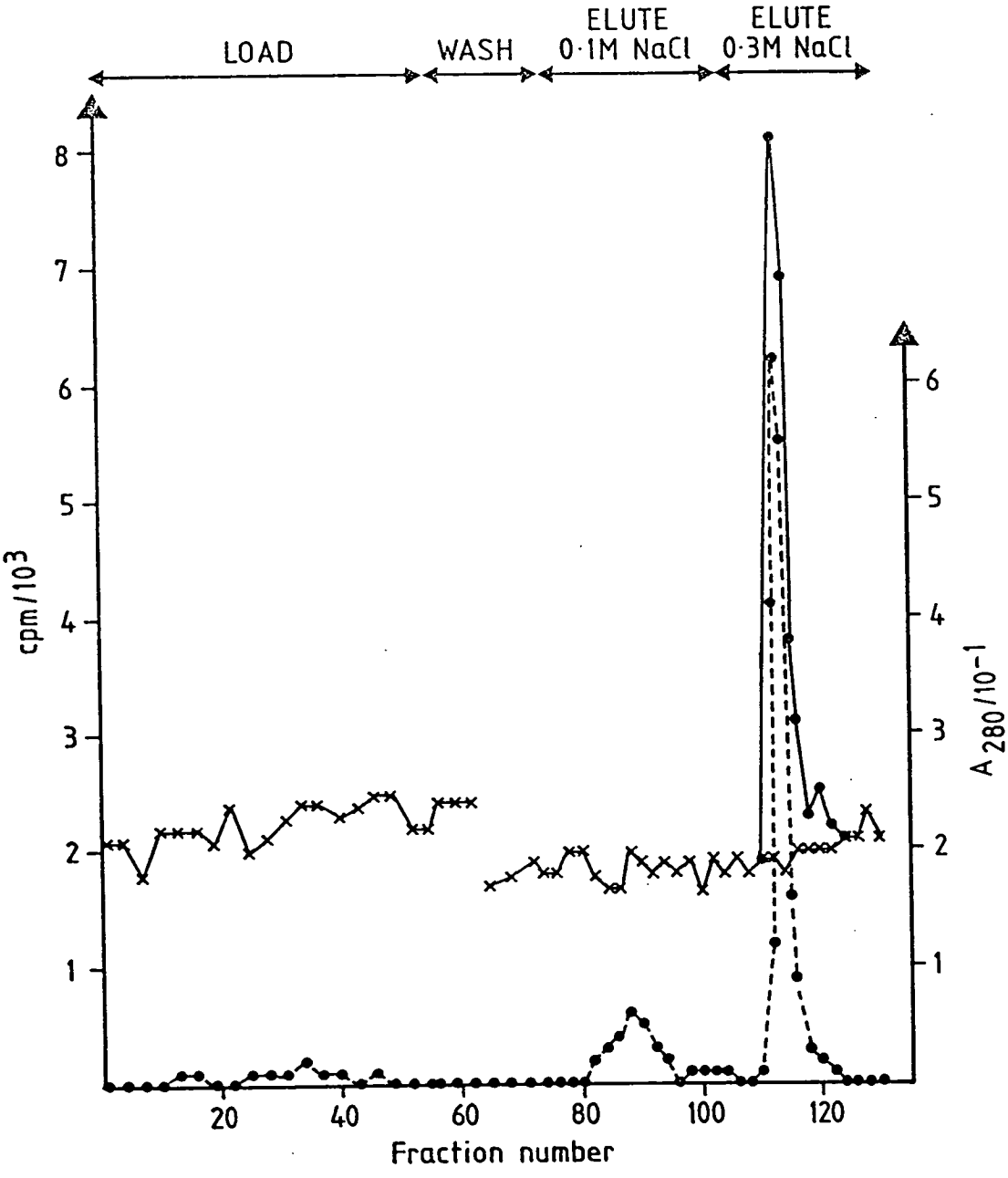
The specific activity of protease La was 1.3mg casein hydrolysed h^{-1} mg^{-1} enzyme (Table 2.3). After storage at -70°C for 15 months, the specific activity had decreased to 0.44mg casein hydrolysed h^{-1} mg^{-1} enzyme (Table 2.4). The variability of the assay of proteolytic activity was estimated to range between 5.21-8.88% (Table 2.4).

FIGURE 2.5 Phosphocellulose Chromatography of an *E. coli*/pJMC40 Cell-Free Extract



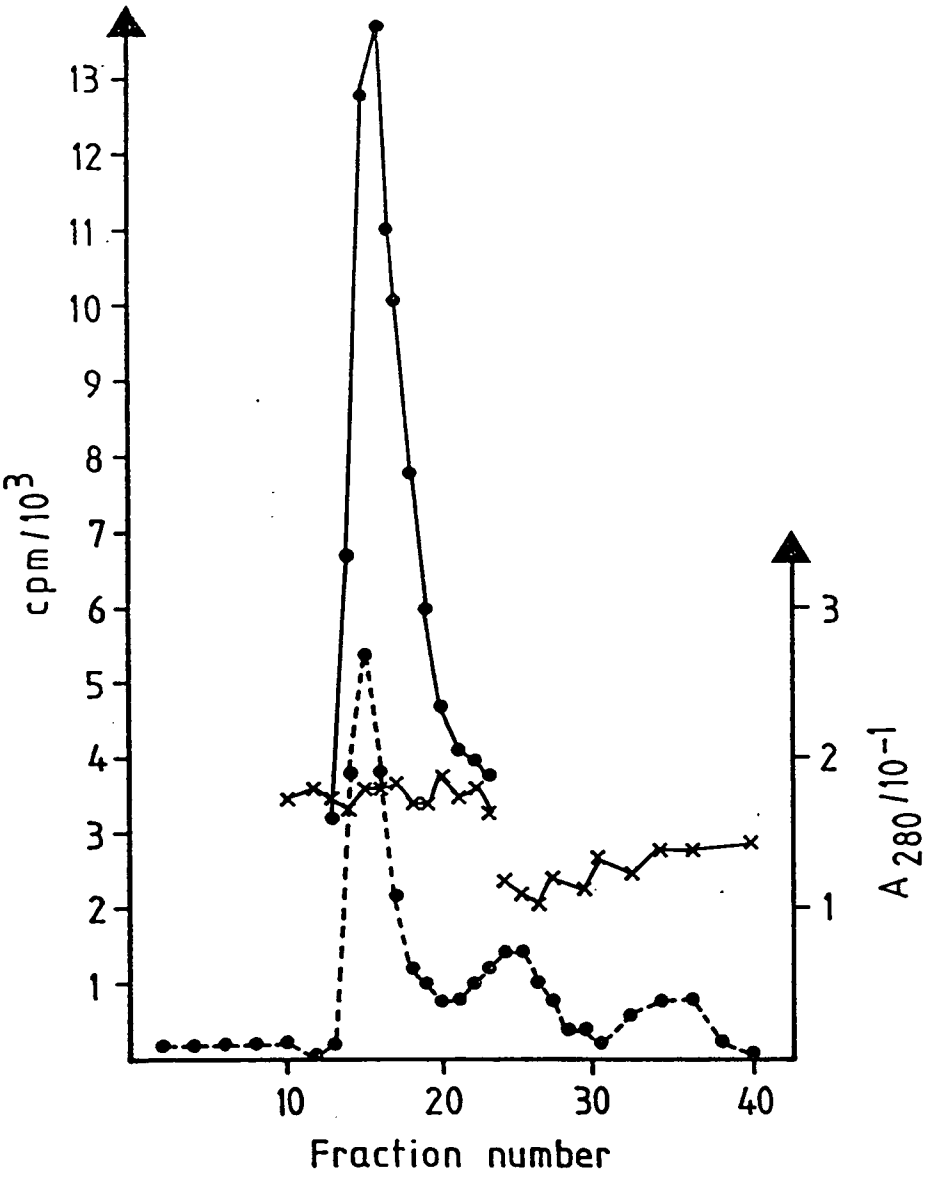
Fractions of 10ml were collected and assayed in the presence and absence of ATP (final concentration 3mM). Assays contained 6µg [^{14}C]-β-lactoglobulin (1.9 x 10^7cpm mg^{-1}), 25mM Tris/HCl pH 8.0, 15mM $MgCl_2$, and 200µl of each fraction. After incubation at 37°C for 90min, acid-soluble radioactivity was determined. Degradation of [^{14}C]-β-lactoglobulin measured in the presence (●——●) or absence (×——×) of ATP compared with A_{280} (●- - -●). For clarity, assays in which no stimulation by ATP was observed have been omitted.

FIGURE 2.6 DEAE-Cellulose Chromatography of Active Fractions From Phosphocellulose Column



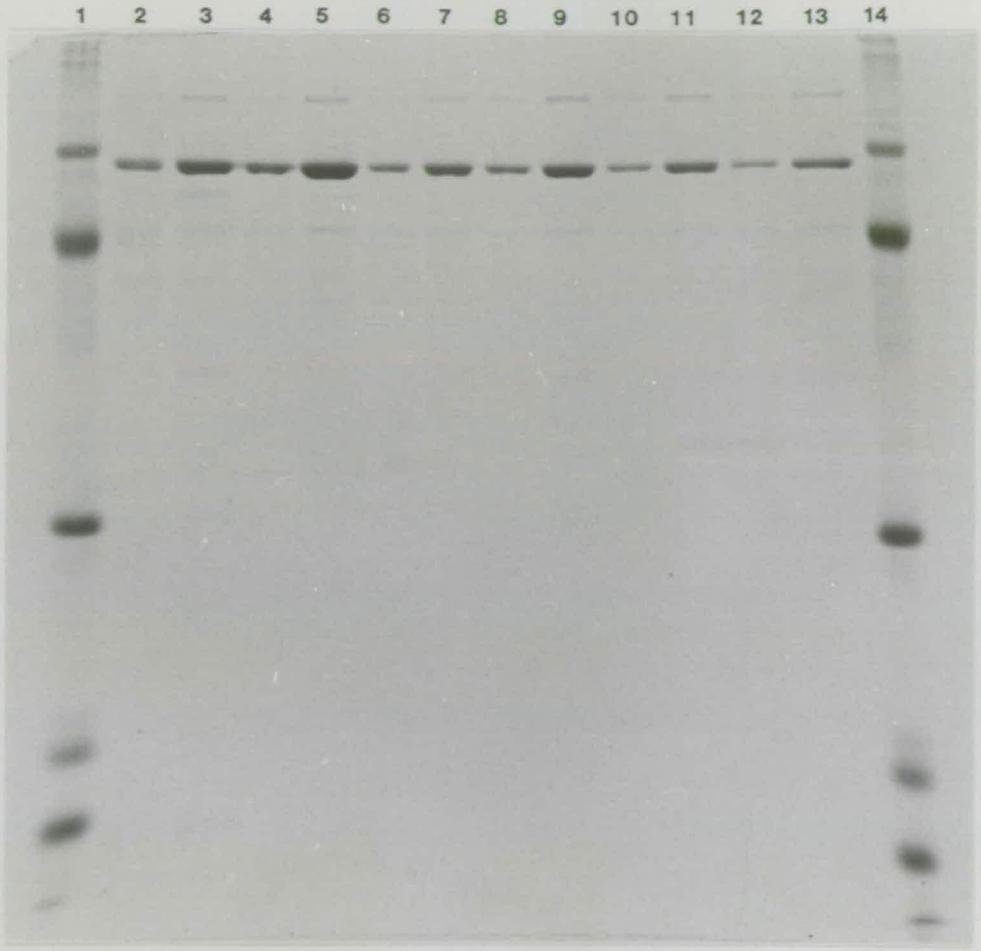
Fractions of 5ml were collected and assayed as described in the legend to Figure 2.5, except that 100µl of each fraction was used and incubation was for 1h. Degradation of [^{14}C]-β-lactoglobulin measured in the presence (●——●) or absence (×——×) of ATP compared with A_{280} (●- - -●). Fractions were assayed with different preparations of [^{14}C]-β-lactoglobulin (fractions 1 to 62 with one batch; 65 to 130 another) which differed in the amount of acid-soluble radioactivity present. For clarity, assays in which no stimulation by ATP was observed have been omitted.

FIGURE 2.7 Gel Filtration Chromatography of Active Fractions From DEAE-Cellulose Column



Fractions of 2ml were collected and assayed as described in the legend to Figure 2.5, except that 20µl of each fraction was used and incubation was for 1h. Degradation of [^{14}C]-β-lactoglobulin measured in the presence (●——●) or absence (×——×) of ATP compared with A_{280} (●- - -●). Fractions were assayed with different preparations of [^{14}C]-β-lactoglobulin (fraction 10 to 23 with one batch; 24 to 40 another) which differed in the amount of acid-soluble radioactivity present. For clarity, assays in which no stimulation by ATP was observed have been omitted.

FIGURE 2.8 Purity of Active Fractions After Gel Filtration Chromatography



Samples of active fractions eluted from the Sephacryl S-300 column were analysed by SDS-PAGE using a 10% gel, which was stained with Coomassie Blue.

Lanes 1 & 14: 3µg each of phosphorylase b, 94,000; BSA, 67,000; β-lactamase, 28,000; β-lactoglobulin, 18,000; cytochrome c, 12,000.

Lanes 2 & 3: 1.5µg and 4µg, fraction 14.

Lanes 4 & 5: 2µg and 5µg, fraction 15.

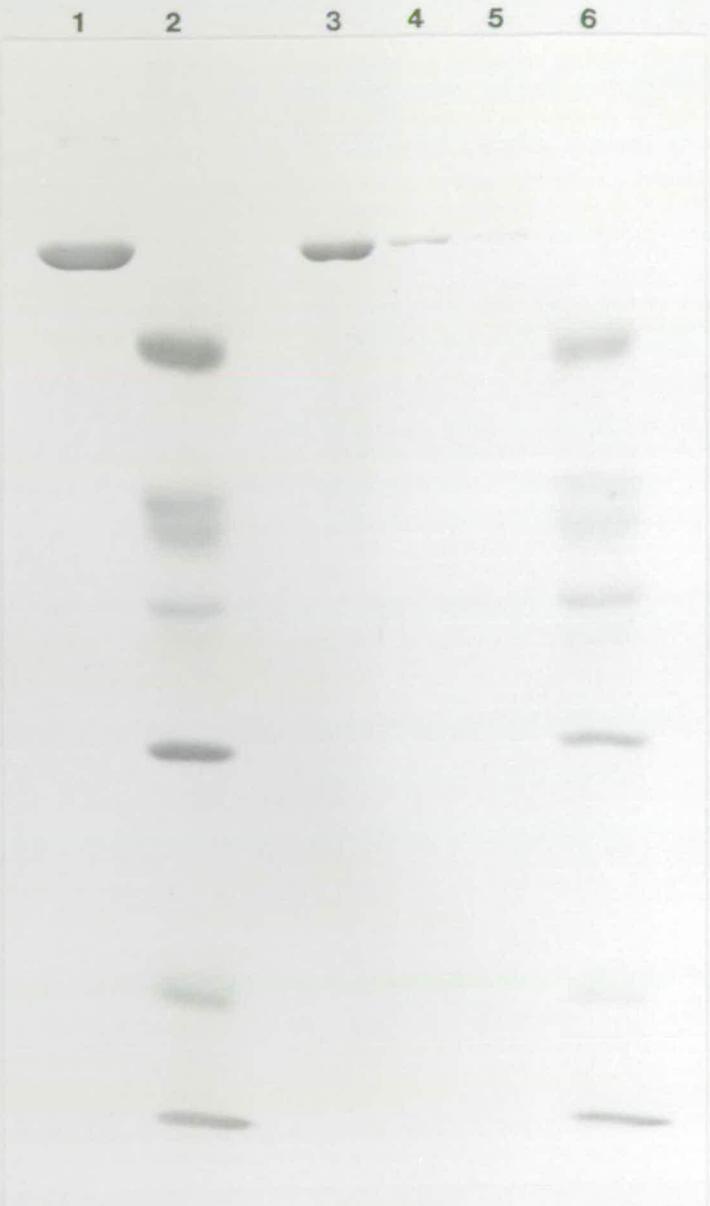
Lanes 6 & 7: 0.5µg and 1.5µg, fraction 16.

Lanes 8 & 9: 1.5µg and 4µg, fraction 17.

Lanes 10 & 11: 0.7µg and 2µg, fraction 18.

Lanes 12 & 13: 0.7µg and 2µg, fraction 19.

FIGURE 2.9 Purity of the Protease La Preparation



Samples of the protease La preparation were analysed by SDS-PAGE using a 10% gel, which was stained with Coomassie Blue.

Lanes 1, 3, 4, & 5: 14µg, 7µg, 1.5µg, and 0.5µg protease La preparation, respectively.

Lanes 2 & 6: 5µg each of BSA, 67,000; egg albumin, 45,000; GAPDH, 36,000; carbonic anhydrase, 29,000; trypsin inhibitor, 20,000; α-lactalbumin, 14,000.

TABLE 2.3 Proteolytic Activity of Protease La Preparation

[^{14}C]-PROTEIN	PERCENTAGE RADIOLABEL ACID-SOLUBLE		
	+ PROTEASE La		- PROTEASE La
	+ ATP	- ATP	
EXPERIMENT 1 [a]			
a. β-lactoglobulin	16.2, 21.0	4.97, 5.30	3.71, 4.97
b. casein	29.4, 29.3	8.90, 10.0	10.8, 8.53
c. α-lactalbumin	42.3, 39.8	8.66, 8.68	8.16, 8.50
d. β-lactamase	16.8, 17.4	17.0, 16.1	14.8, 16.2
e. oxidized-β-lactoglobulin	19.8, 21.3, 20.8	9.49, 8.88, 9.38	9.79, 8.49, 8.39
EXPERIMENT 2 [b]			
casein	33.1, 36.4, 30.3	12.5, 11.6, 13.1	12.5, 12.2, 12.5
α-lactalbumin	22.2, 24.5, 25.3	8.42, 8.71, 8.77	8.14, 8.13, 8.18
β-lactamase	14.2, 14.6, 14.0	12.0, 12.8, 12.8	12.5, 13.4, 12.8
oxidized-β-lactoglobulin	38.8, 39.0, 35.6	18.1, 18.9, 18.1	16.5, 16.4, 17.1

AMOUNT OF PROTEASE La PREPARATION (μg)	PERCENTAGE RADIOLABEL ACID-SOLUBLE		
	+ PROTEASE La		- PROTEASE La
	+ ATP	- ATP	
EXPERIMENT 3 [c]			
2.5	57.4, 64.4	18.8, 17.6	18.5, 21.6
0.6	63.1, 56.9	17.3, 18.3	21.6, 20.6
0.15	37.2, 32.0	22.9, 22.2	21.1, 20.2

a. Assays contained 25mM Tris/HCl pH 8.0, 25mM $MgCl_2$, and about 2μg [^{14}C]-protein (0.5-1.5 x 10^7cpm mg^{-1}) in a final volume of 25μl (a & b) or 30μl (c, d, & e). Protease La (enzyme subunit:protein molar ratio of 1:80 (a & b) or 1:40 (c, d, & e)) and ATP (final concentration 3mM) were added as indicated. After incubation at 37°C for 3h (a, b, & e), or 5h (c & d), acid-soluble radioactivity was determined.

b. Assays contained 25mM Tris/HCl pH 8.0, 25mM $MgCl_2$, and about 2μg [^{14}C]-protein (0.5-2.0 x 10^7cpm mg^{-1}) in a final volume of 30μl. Protease La (enzyme subunit:protein molar ratio of 1:40) and ATP (final concentration 3mM) were added as indicated. After incubation at 37°C for 3h, acid-soluble radioactivity was determined.

c. Assays contained 30mM Tris/HCl pH 8.0, 15mM $MgCl_2$, and about 2μg [^{14}C]-α-lactalbumin (1.3 x 10^7cpm mg^{-1}) in a final volume of 30μl. Varying amounts of protease La (enzyme subunit: α-lactalbumin molar ratios of 1:5, 1:20, and 1:80) and ATP (final concentration 3mM) were added as indicated. After incubation at 37°C for 100min, acid-soluble radioactivity was determined.

TABLE 2.4 Variability of the Assay Procedure for Protease La Activity

ADDITIONS	PERCENTAGE RADIOLABEL ACID-SOLUBLE	
	β-LACTOGLOBULIN [a]	CASEIN [b]
- protease La, - ATP	4.75 ±0.289 (6.08%)	4.13 ±0.256 (6.20%)
+ protease La, - ATP	4.64 ±0.412 (8.88%)	4.55 ±0.237 (5.21%)
- protease La, + ATP	4.61 ±0.269 (5.84%)	4.42 ±0.386 (8.73%)
+ protease La, + ATP	9.77 ±0.654 (6.69%)	14.1 ±0.920 (6.52%)

a. Assays contained 4μg [^{14}C]-β-lactoglobulin (1.1 x 10^7cpm mg^{-1}), 25mM Tris/HCl pH 8.0, and 25mM $MgCl_2$, in a final volume of 30μl. Protease La (0.42μg, enzyme subunit:protein molar ratio of 1:40) and ATP (final concentration 3mM) were added as indicated. After incubation at 37°C for 2h, acid-soluble radioactivity was determined. Values presented are means ±S.D. (n=13). The variability of the assay is shown in parentheses.

b. Assays were as above except that [^{14}C]-casein (6 x 10^6cpm mg^{-1}) and 0.39μg protease La were used.

2.2 SPECIFICITY OF THE *E. coli* ATP-DEPENDENT PROTEASE La

2.2.1 Determination of Optimum Conditions for the Activity of Protease La

Conditions which gave the greatest recovery of acid-soluble radioactivity when [^{14}C]-β-lactoglobulin was incubated with protease La were determined (Figure 2.10). In agreement with the results of Larimore *et al.* (1982), proteolysis occurred at a linear rate for up to 3h (Figure 2.10.F) and was proportional to enzyme concentration over a wide range (Figure 2.10.A). Increasing the concentration of [^{14}C]-β-lactoglobulin (by reducing the assay volume) led to a greater rate of proteolysis until saturation was attained (Figure 2.10.H and inset). The apparent K_m of [^{14}C]-β-lactoglobulin is about 2μM (Figure 2.10.H) which is in reasonable agreement with the apparent K_m of [^{3}H]-casein (0.5-2.0μM) reported by Larimore *et al.* (1982). Protease La was found to have a low ionic strength requirement (Figure 2.10.C). The pH and concentrations of ATP and $MgCl_2$ which gave the greatest rate of proteolysis (Figures 2.10.B, 2.10.E, and 2.10.G) were essentially the same as those reported when [^{3}H]-casein was used as a substrate (Waxman and Goldberg, 1985; Larimore *et al.*, 1982). Addition of single-stranded DNA resulted in almost a two-fold decrease in the rate of proteolysis of [^{14}C]-β-lactoglobulin by protease La (Figure 2.10.D). Further experiments to investigate the effect of DNA on the activity of protease La are described in Section 2.3.

FIGURE 2.10 Effect of Varying Conditions on Protease La Activity

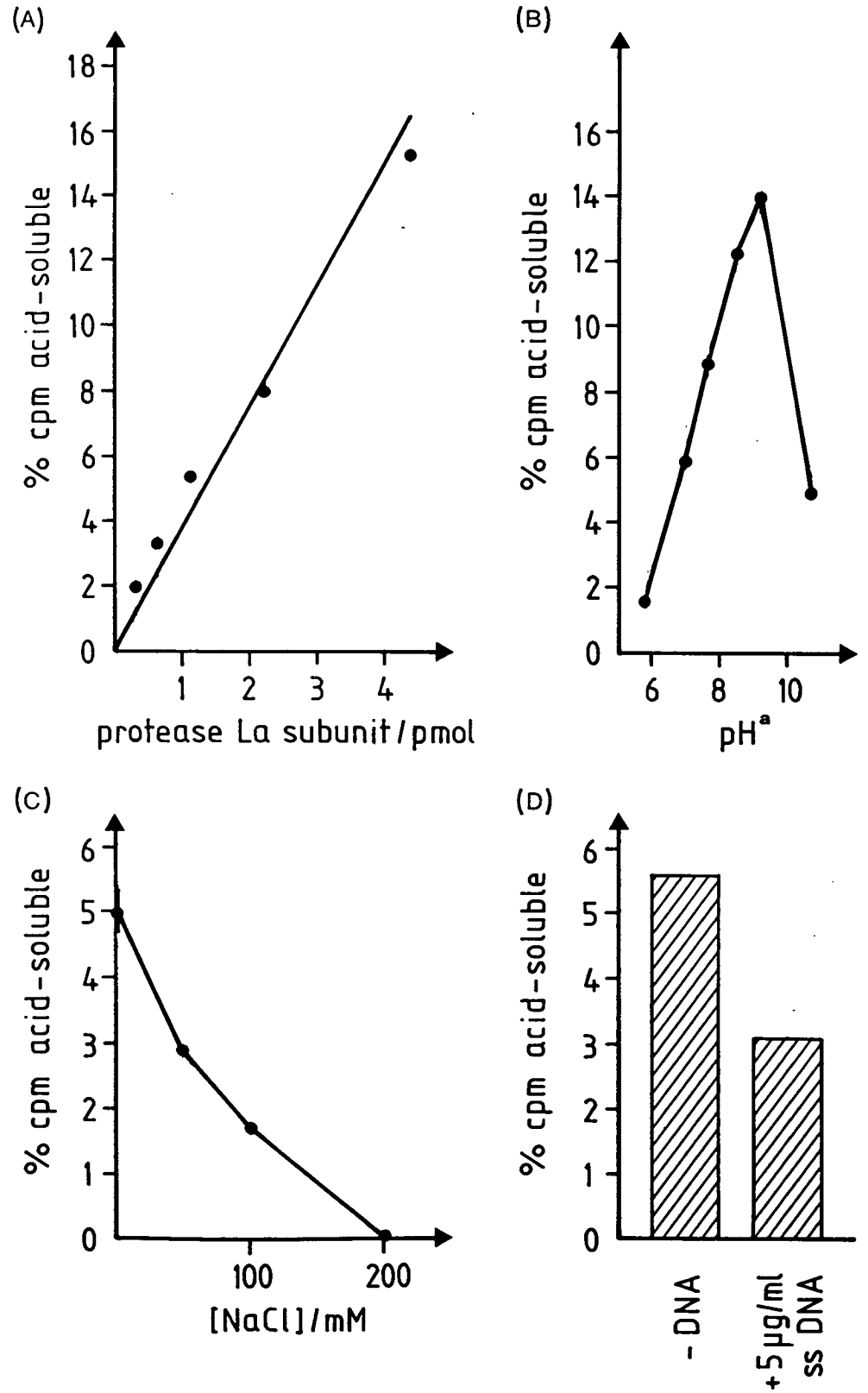
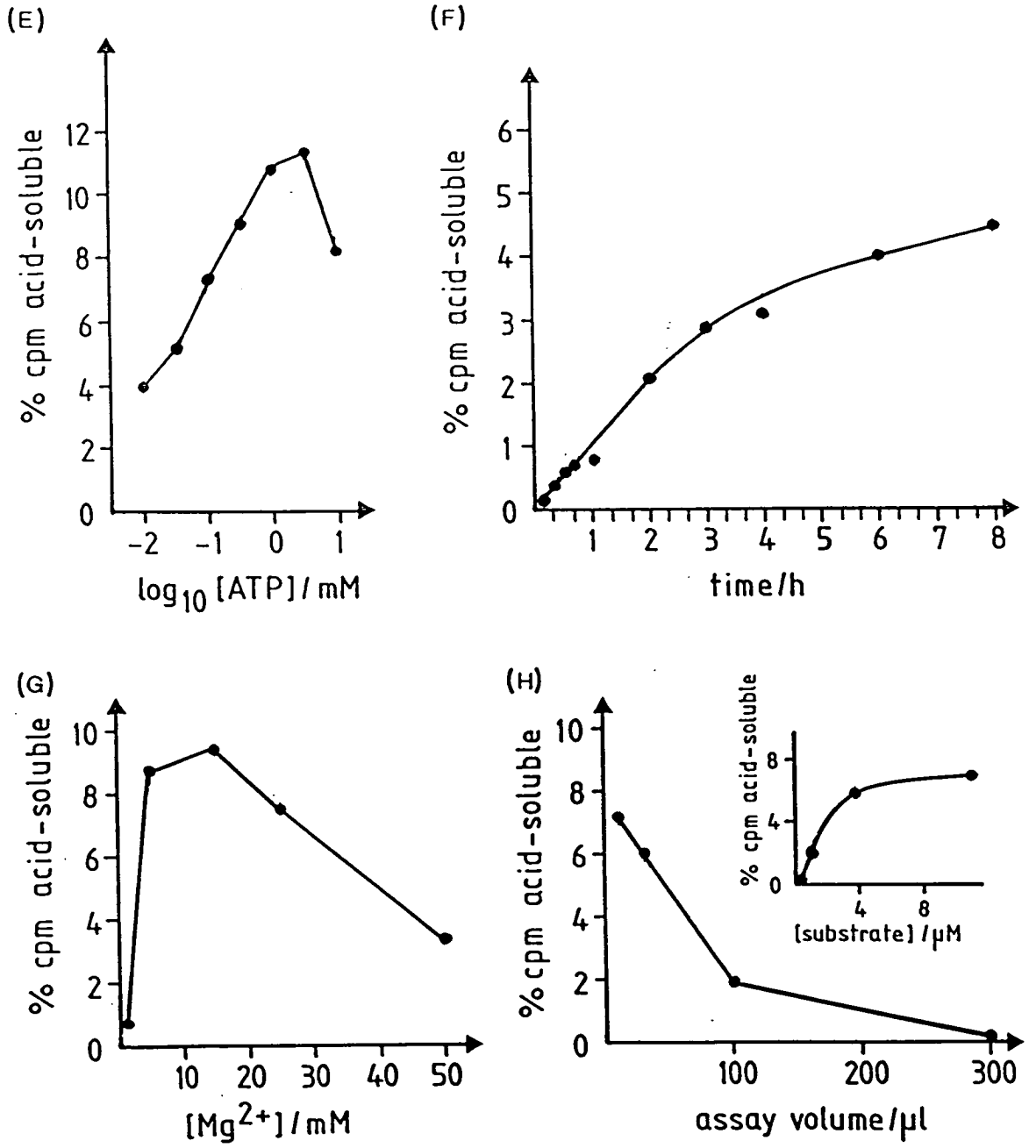


FIGURE 2.10 (continued)



Unless stated otherwise each assay was incubated at 37°C for 3h and contained: 2µg [^{14}C]-β-lactoglobulin (0.11nmol), 50mM Tris/HCl pH 8.0, 25mM $MgCl_2$, 3mM ATP, and 0.13µg protease La (1.4pmol subunit) in a final volume of 30µl. Acid-soluble radioactivity was determined and the values shown are the mean of triplicate assays, corrected for acid-soluble radioactivity in the control (no enzyme).

a Buffers used (50mM; pH values shown at 37°C): Piperazine pH 5.8; HEPES pH 7.0; Tris/HCl pH 7.7; BICINE pH 8.5; Borate pH 9.2; Na_2CO_3 pH 10.7.

FIGURE 2.11 Digestion of [^{14}C]-Proteins by Protease La

For each [^{14}C]-protein, three reaction mixtures containing 50mM Tris/HCl pH 8.0, 25mM $MgCl_2$ and about 60µg of the radiolabelled protein, in a final volume of 150µl, were prepared. Protease La (at an enzyme subunit:protein molar ratio of 1:80) and ATP (final concentration 3mM) were added to one mixture; protease La was added to another; and to the remaining mixture, ATP was added. After incubation at 37°C for 16h, 25µl aliquots were removed and treated as described in the following sections.

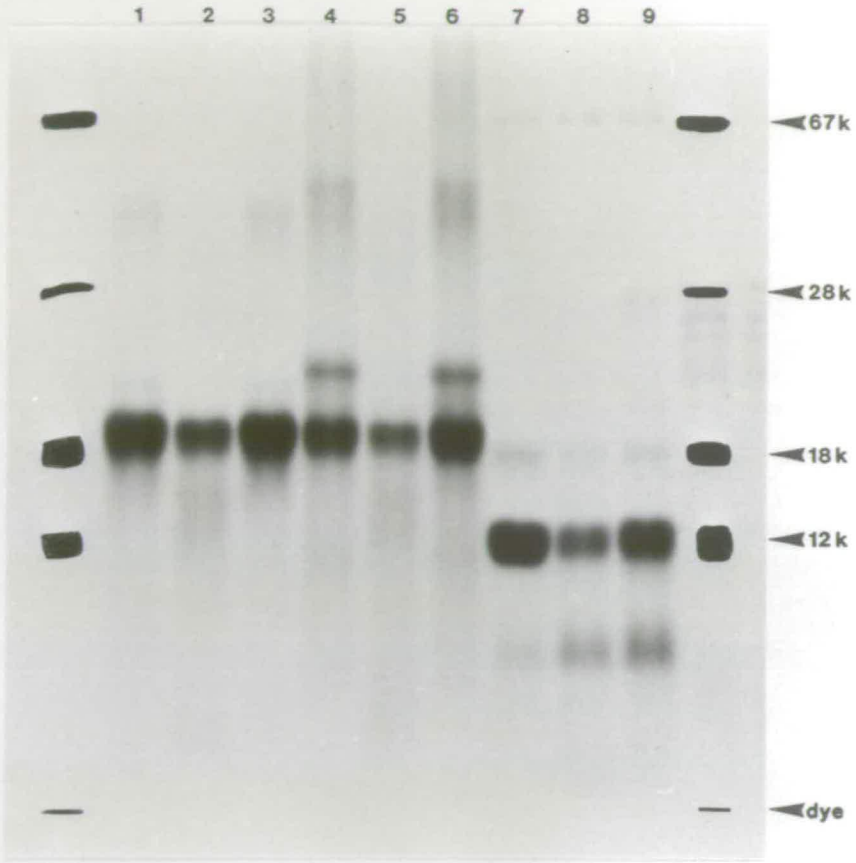
1 Degradation of [^{14}C]-Proteins to Acid-Soluble Peptides

[^{14}C]-PROTEIN	SPECIFIC ACTIVITY (cpm mg^{-1})	PERCENTAGE RADIOLABEL ACID-SOLUBLE [a]	
		+ ATP	- ATP
β-lactoglobulin	1.3×10^7	31	3
oxidized-β-lactoglobulin	1.7×10^7	33	2
α-lactalbumin	1.3×10^7	26	-3

a. BSA and TCA were added to the aliquots and the acid-soluble radioactivity was determined. Values shown are corrected for acid-soluble radioactivity present in the control (no enzyme).

FIGURE 2.11 (continued)

2 Degradation of [^{14}C]-Proteins Revealed by SDS-PAGE



Sample buffer was added to the aliquots and they were analysed by SDS-PAGE using a 12% gel, which was stained by the method of Steck et al. (1980) and exposed to X-ray film to produce an autoradiograph. The positions of molecular weight markers and the dye front are indicated: BSA, 67,000; β-lactamase, 28,000; β-lactoglobulin, 18,000; cytochrome c, 12,000.

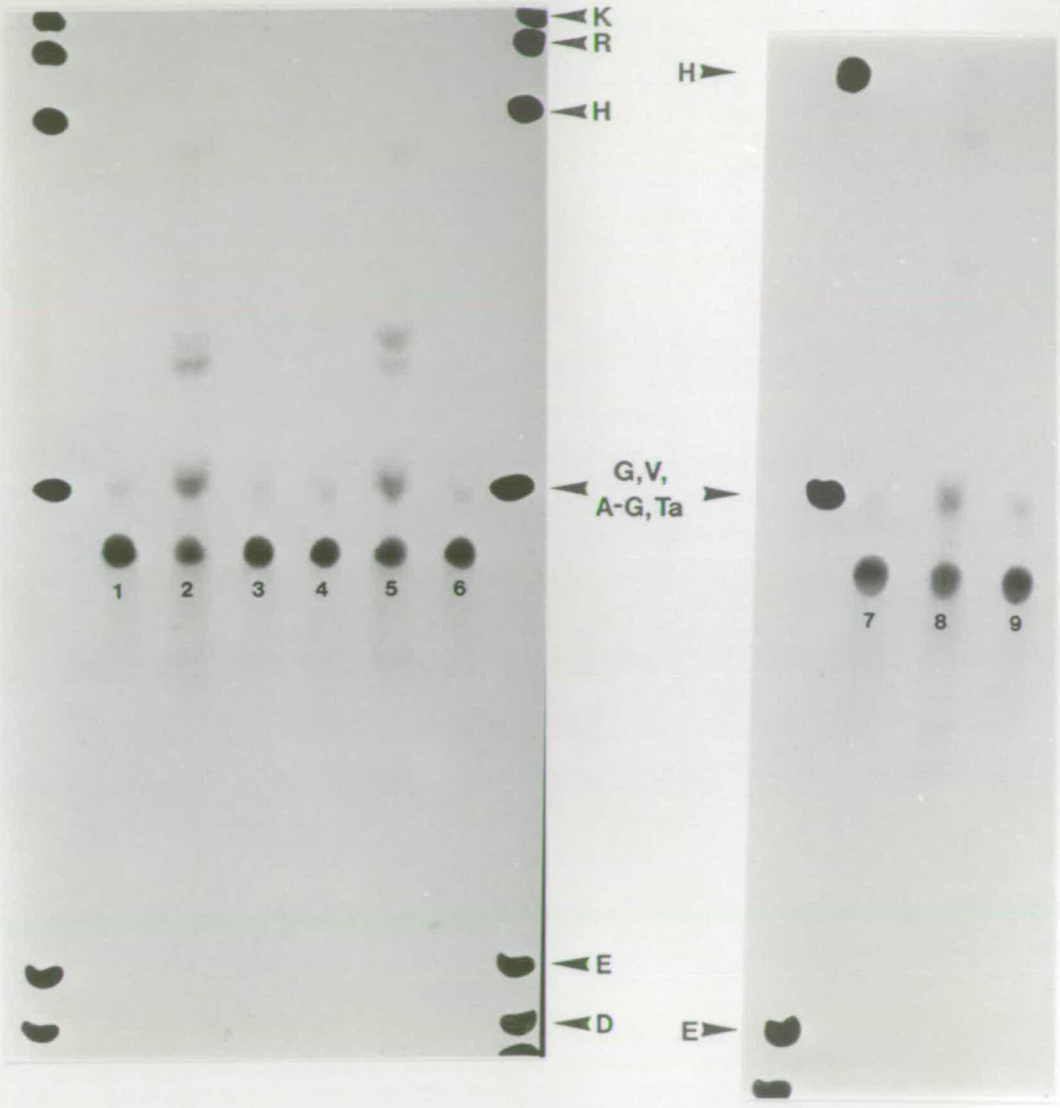
Lanes 1-3: [^{14}C]-β-lactoglobulin + ATP; + protease La + ATP; & + protease La, respectively.

Lanes 4-6: [^{14}C]-oxidised-β-lactoglobulin + ATP; + protease La + ATP; & + protease La, respectively.

Lanes 7-9: [^{14}C]-α-lactalbumin + ATP; + protease La + ATP; & + protease La, respectively.

FIGURE 2.11 (continued)

3 Degradation of [^{14}C]-Proteins Revealed by HVPE



The aliquots were dried, redissolved in 0.1N NH_4OH and electrophoresed on Whatman 3MM paper at pH 6.5. Papers were stained and exposed to X-ray film to produce an autoradiograph. The positions of amino acid standards are indicated. Ta: taurine.

Lanes 1-3: [^{14}C]-β-lactoglobulin + ATP; + protease La + ATP; & + protease La, respectively.

Lanes 4-6: [^{14}C]-oxidised-β-lactoglobulin + ATP; + protease La + ATP; & + protease La, respectively.

Lanes 7-9: [^{14}C]-α-lactalbumin + ATP; + protease La + ATP; & + protease La, respectively.

2.2.2 Degradation of [^{14}C]-Proteins by Protease La

Goldberg (1985) reported that protease La digests radiolabelled proteins to acid-soluble peptides with molecular weights greater than 1500. The number and nature of peptides produced when [^{14}C]-proteins were degraded by protease La was investigated (Figure 2.11). After incubation with protease La, about one third of the radiolabel of each [^{14}C]-protein was rendered acid-soluble in the presence of ATP, whereas only a small recovery was achieved in its absence (Figure 2.11.1). A decrease in the amount of radiolabelled protein was detected by SDS-PAGE only after incubation with protease La and ATP (Figure 2.11.2). Discrete degradation products of [^{14}C]-β-lactoglobulin and [^{14}C]-oxidized-β-lactoglobulin were not detected, but a degradation product of [^{14}C]-α-lactalbumin was detected by SDS-PAGE (Figure 2.11.2). This polypeptide (molecular weight about 11,000) was found in all the reaction mixtures of [^{14}C]-α-lactalbumin, although the amount present varied (Figure 2.11.2).

The small amount of the polypeptide in the reaction mixture which lacked protease La may have been caused by the labelling procedure. Reductive methylation of proteins has been reported to cause peptide bond cleavage, although substitution of sodium borohydride by sodium cyanoborohydride should have prevented this (Jentoft and Dearborn, 1979). However, the presence of high molecular weight radiolabelled proteins in the preparations suggests that labelling conditions were not ideal (Figure 2.11.2). Other possible explanations for the presence of the polypeptide are: contamination of α-lactalbumin by a protease which has a similar specificity to protease La (or a protease contaminant of the protease La preparation); or contamination of the α-lactalbumin by a protein of molecular weight about 11,000, which was radiolabelled.

The greatest amount of the polypeptide was detected in the reaction mixture which contained protease La, but lacked ATP (Figure 2.11.2). Possible explanations for this unexpected finding are:

1. The proteolytic activity of protease La is stimulated by and not dependent upon ATP.
2. The protease La preparation is contaminated by a protease (which degrades [^{14}C]-α-lactalbumin to a polypeptide of molecular weight ≈11,000). If this is the case, then the polypeptide must be a substrate of protease La since less was present in the reaction mixture which contained protease La and ATP.
3. The [^{14}C]-α-lactalbumin preparation contains a factor (possibly ATP) which supports limited digestion by protease La.

Smaller amounts of both the polypeptide and labelled α-lactalbumin were detected in the reaction mixture which contained protease La and ATP, compared with the reaction mixture which contained protease La but lacked ATP (Figure 2.11.2). This finding suggests that this polypeptide is not an end-product of the digestion of [^{14}C]-α-lactalbumin by protease La. Possibly, degradation of this polypeptide is the rate-limiting step. However, this hypothesis relies upon two assumptions: that the protease La preparation is free from contamination by proteases; and that the labelling procedure produced a homogeneous preparation of [^{14}C]-α-lactalbumin.

After incubation peptides were detected by HVPE in all the reaction mixtures, for each of the [^{14}C]-proteins (Figure 2.11.3). Small amounts of peptides of similar mobilities were found in the controls; that is the reaction mixtures which did, and did not, contain protease La. In the cases of [^{14}C]-β-lactoglobulin and [^{14}C]-oxidized-β-lactoglobulin these peptides were probably contaminants of the labelled preparations since the amounts of the peptides in the reaction mixtures were similar. However, in the reaction mixture of [^{14}C]-α-lactalbumin which contained protease La, greater amounts of peptides were detected than in the reaction mixture which lacked protease La. This correlates with the results obtained when reaction mixtures of [^{14}C]-α-lactalbumin were analysed by SDS-PAGE (Figure 2.11.2): with both techniques greater degradation was detected in the presence of protease La (but without ATP) than in its absence. Possible explanations for this unexpected finding were given above. However, if the protease La preparation is contaminated by a protease then it must be a highly specific protease because neither degradation of [^{14}C]-β-lactoglobulin nor [^{14}C]-oxidized-β-lactoglobulin in the presence of protease La (without ATP) was detected by either technique.

Several additional peptides of widely different mobilities were detected by HVPE in the reaction mixtures which contained protease La and ATP, for each of the [^{14}C]-proteins (Figure 2.11.3). Incubation of [^{14}C]-β-lactoglobulin and of [^{14}C]-oxidized-β-lactoglobulin with protease La and ATP produced peptides of identical mobilities, suggesting that oxidation of β-lactoglobulin prior to radiolabelling did not influence which peptide bonds were hydrolysed by protease La.

It was surprising that digestion of [^{14}C]-α-lactalbumin by protease La, in the absence of ATP, was not revealed by the recovery of acid-soluble radioactivity (Figure 2.11.1). This can be explained partly by the discovery that the degradation product of molecular weight about 11,000 was insoluble in 10% TCA (Figure 2.12.1). However,

1 Three reaction mixtures containing: 30mM Tris/HCl pH 8.0, 15mM $MgCl_2$, and about 20μg [^{14}C]-α-lactalbumin (1.3 x 10^7 cpm mg^{-1}), in a final volume of 50μl, were prepared. ATP (final concentration 3mM) was added to one reaction mixture; protease La (enzyme subunit:protein molar ratio of 1:80) was added to another; and protease La and ATP were added to the remaining mixture. After incubation at 37°C for 14h, the reaction mixtures were divided into two. One aliquot was analysed directly by SDS-PAGE using a 15% gel (A). To the other aliquot, BSA (final concentration 2mg ml^{-1}) and TCA (final concentration 10%) were added. After incubation on ice for 30min and centrifugation (11,600g_n for 10min), the acid-insoluble and acid-soluble material were neutralized and then analysed by SDS-PAGE using a 15% gel (B & C). The gels were stained by the method of Steck et al. (1980) and also exposed to X-ray film to produce an autoradiograph. The positions of molecular weight markers (BSA, 67,000; β-lactamase, 28,000; β-lactoglobulin, 18,000; cytochrome c, 12,000) are indicated.

Lane 1 (A, B, & C): reaction mixture + ATP only.

Lane 2 (A, B, & C): reaction mixture + protease La + ATP.

Lane 3 (A, B, & C): reaction mixture + protease La only.

2 Three reaction mixtures containing: 50mM Tris/HCl pH 8.0, 25mM $MgCl_2$, and about 10μg [^{14}C]-α-lactalbumin (1.3 x 10^7 cpm mg^{-1}), in a final volume of 25μl, were prepared. ATP (final concentration 3mM) was added to one reaction mixture; protease La (enzyme subunit:protein molar ratio of 1:80) was added to another; and protease La and ATP were added to the remaining mixture. Following incubation at 37°C for 16h, BSA (final concentration 2mg ml^{-1}) and TCA (final concentration 10%) were added to the reaction mixtures. After incubation on ice for 30 minutes and centrifugation (11,600g_n for 10min) the acid-soluble material was treated by the method of Harris and Hindley (1965) to remove the TCA prior to electrophoresis on Whatman 3MM paper at pH 6.5. The paper was stained and exposed to X-ray film to produce an autoradiograph. The positions of amino acid standards are indicated. Ta: taurine.

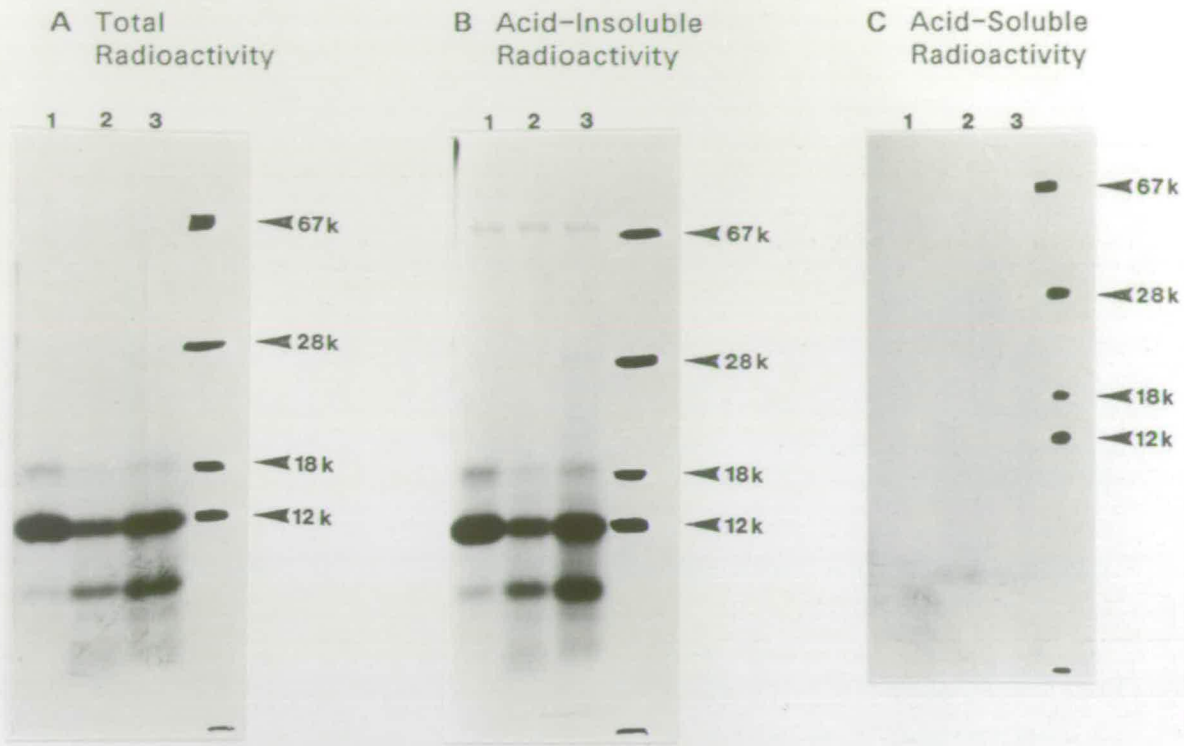
Lane 1: reaction mixture + ATP only.

Lane 2: reaction mixture + protease La + ATP.

Lane 3: reaction mixture + protease La only.

FIGURE 2.12 Degradation of [^{14}C]-α-Lactalbumin by Protease La

1 Analysed by SDS-PAGE



2 Analysed by HVPE

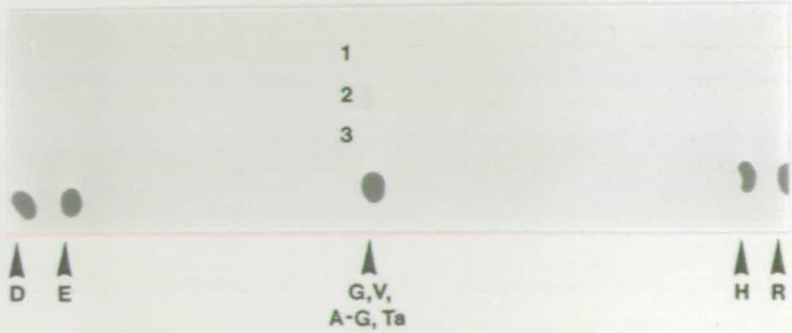


TABLE 2.5 Acid-Soluble Radioactivity Present in Reaction Mixtures of [^{14}C]-α-Lactalbumin

REACTION MIXTURE [a]	cpm 0.4ml^{-1}	PERCENTAGE RADIOLABEL ACID-SOLUBLE
- protease La - ATP	1573, 1572, 1581	8.14, 8.13, 8.18
+ protease La - ATP	1628, 1683, 1696	8.42, 8.71, 8.77
+ protease La + ATP	4288, 4732, 4885	22.2, 24.5, 25.3

a. Reaction mixtures contained 50mM Tris/HCl pH 8.0, 25mM $MgCl_2$, and about 2μg [^{14}C]-α-lactalbumin (1.5 x 10^7cpm mg^{-1}) in a volume of 30μl. Protease La (enzyme subunit:protein molar ratio of 1:40) and ATP (final concentration 3mM) were added as indicated. Reaction mixtures were incubated at 37°C for 3h and acid-soluble radioactivity determined.

TABLE 2.6 Non-Hydrolysable Analogues of ATP and Protease La

RIBONUCLEOTIDE	CONCENTRATION (mM)	PERCENTAGE RADIOLABEL ACID-SOLUBLE		
		α-LACTALBUMIN	OXIDIZED-β-LACTOGLOBULIN	CASEIN
ATP	3.0	18.8, 19.2, 20.0	19.6, 20.0, 23.5	22.8, 18.7, 17.9
	0.3	19.0, 19.6, 17.4	22.2, 19.2, 19.6	23.0, 22.3, 20.9
	0.03	10.8, 9.77, 9.49	10.0, 8.46, 10.0	13.7, 12.7, 13.5
AMP-PCP	3.0	7.99, 7.18, 6.97	8.81, 6.71, 7.95	8.37, 7.55, 7.34
	0.3	7.45, 7.24, 7.51	9.09, 7.14, 8.31	7.12, 7.02, 6.91
	0.03	7.83, 8.05, 7.71	8.55, 7.39, 7.65	8.11, 8.28, 6.91
AMP-PNP	3.0	7.79, 7.61, 8.74	9.20, 9.09, 8.38	8.20, 9.36, 8.69
	0.3	6.42, 8.20, 8.17	8.68, 7.82, 8.08	7.56, 7.23, 7.73
	0.03	6.87, 6.97, 7.85	8.01, 7.83, 8.55	8.46, 7.99, 8.15
- Protease La		7.31, 6.69, 7.49	7.30, 7.21, 7.28	7.45, 8.07, 7.26

a. Assays contained 25mM Tris/HCl pH 8.0, 25mM $MgCl_2$, about 2μg [^{14}C]-protein (1-2 x 10^7cpm mg^{-1}), and protease La (enzyme subunit:protein molar ratio of 1:40) in a final volume of 30μl. Varying concentrations of ATP, AMP-PCP, and AMP-PNP were added. After incubation at 37°C for 3h, acid-soluble radioactivity was determined.

smaller degradation products (sum of molecular weights about 3,000) should have been soluble in 10% TCA. Indeed, an acid-soluble peptide was detected by SDS-PAGE (Figure 2.12.1) although not by HVPE (Figure 2.12.2). However, when treating the samples to remove TCA prior to HVPE, acid-soluble peptides may have been lost. Evidence to support this suggestion is given by the finding that the amounts of peptides detected in reaction mixtures containing protease La and ATP are very different before and after treatment to remove TCA (Figures 2.11.3 and 2.12.2). Consequently, determination of the amount of acid-soluble radioactivity present in reaction mixtures of [^{14}C]-α-lactalbumin was repeated (Table 2.5). Digestion of [^{14}C]-α-lactalbumin by protease La in the absence of ATP was detected, but the recovery of acid-soluble radioactivity was very low. Presumably, the sensitivity of the previous determination of acid-soluble radioactivity in the reaction mixtures was insufficient to detect such a low level of proteolytic activity (Figure 2.11.1).

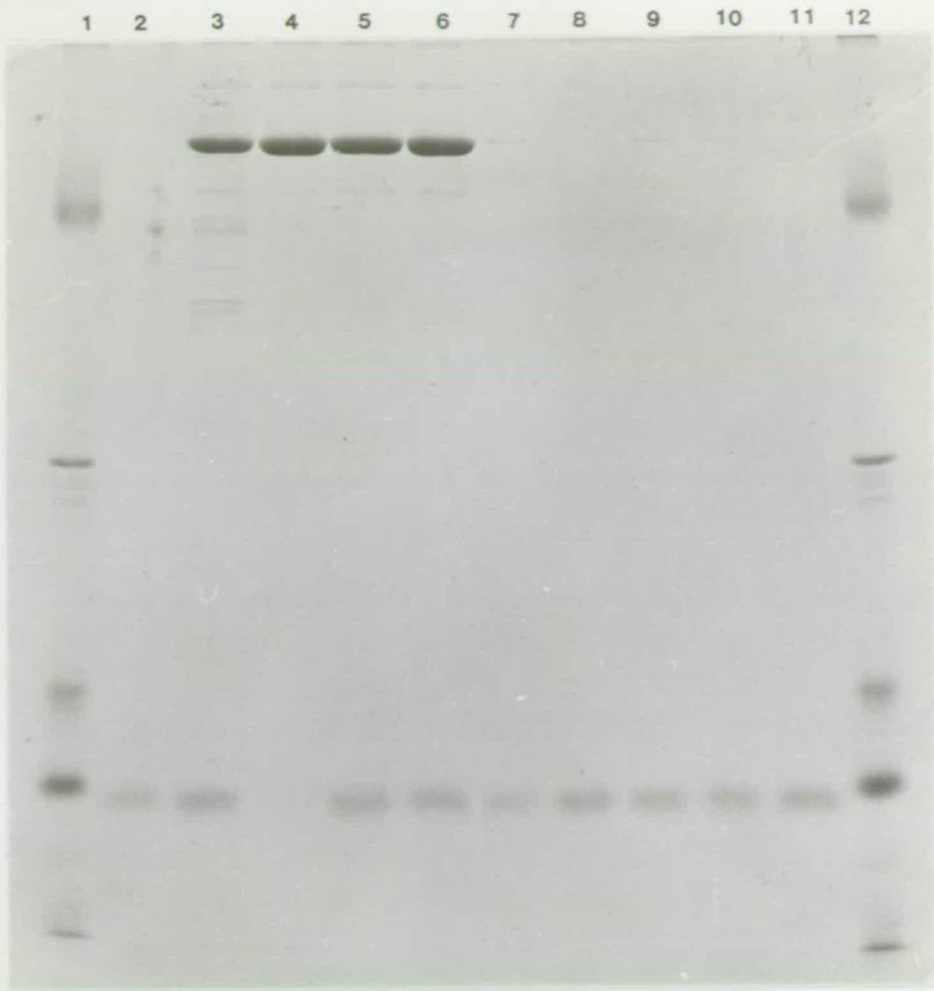
A range of [^{14}C]-proteins were incubated with protease La in the presence of ATP, or non-hydrolysable analogues of ATP: compared to the release of acid-soluble radioactivity with ATP, negligible release occurred with the non-hydrolysable analogues (Table 2.6). Increasing the amount of protease La (from a molar ratio of 1:40 to 1:1)

TABLE 2.7 Protease La and Non-Hydrolysable Analogues of ATP

RATIO PROTEASE La: [^{14}C]-α-LACTALBUMIN	RIBONUCLEOTIDE	PERCENTAGE RADIOLABEL ACID-SOLUBLE
1:40	-	10.6, 10.8, 11.0
1:1	-	11.6, 11.6, 12.0
1:40	ATP	23.7, 24.2, 25.9
1:1	ATP	35.7, 40.7, 41.7
1:40	AMP-PCP	10.2, 11.8, 11.6
1:1	AMP-PCP	11.5, 12.3, 11.8
1:40	AMP-PNP	11.3, 11.9, 11.8
1:1	AMP-PNP	12.5, 13.3, 13.2
- Protease La		9.66, 11.1, 10.9

a. Assays contained 25mM Tris/HCl pH 8.0, 25mM $MgCl_2$, about 2μg [^{14}C]-α-lactalbumin (1.5 x 10^7cpm mg^{-1}) in a final volume of 60μl. Protease La was added at the molar ratios (enzyme subunit: [^{14}C]-α-lactalbumin) indicated. Ribonucleotides were added to a final concentration of 3mM. After incubation at 37°C for 3h, acid-soluble radioactivity was determined.

FIGURE 2.13 Protease La and Non-Hydrolysable Analogues of ATP



Reaction mixtures were incubated at 37°C for 6h and contained 25mM Tris/HCl pH 8.0, 25mM $MgCl_2$ and about 2µg [^{14}C]-α-lactalbumin (1.5 x 10^7 cpm mg^{-1}), in a final volume of 60µl. Protease La and [^{14}C]-α-lactalbumin were added in the molar ratios 1:1 or 1:40 (protease La subunit:[^{14}C]-α-lactalbumin). Ribonucleotides (ATP, AMP-PCP, or AMP-PNP) were added to a final concentration of 3mM. Reaction mixtures were analysed by SDS-PAGE using a 17.5% gel, and the gel was stained with Coomassie Blue.

Lanes 1 and 12: BSA, 67,000; β-lactamase, 28,000; β-lactoglobulin, 18,000; cytochrome c, 12,000.
Lanes 2 & 11: no addition.
Lane 3: protease La (1:1), no ribonucleotide.
Lane 4: protease La (1:1) and ATP.
Lane 5: protease La (1:1) and AMP-PCP.
Lane 6: protease La (1:1) and AMP-PNP.
Lane 7: protease La (1:40) and ATP.
Lane 8: protease La (1:40) and AMP-PCP.
Lane 9: protease La (1:40) and AMP-PNP.
Lane 10: protease La (1:40), no ribonucleotide.

had little effect on the amount of acid-soluble radioactivity released with the non-hydrolysable analogues of ATP (Table 2.7). When degradation of [^{14}C]-α-lactalbumin by protease La in the presence of ATP or non-hydrolysable analogues was monitored by SDS-PAGE, disappearance of the band corresponding to [^{14}C]-α-lactalbumin only occurred in reaction mixtures containing ATP, irrespective of the amount of protease La (Figure 2.13). Furthermore, bands corresponding to high molecular weight degradation products were not observed in any of the reaction mixtures (Figure 2.13).

2.2.3 Degradation of Native Proteins by Protease La

2.2.3.1 Purification of Wild-Type and Mutant Forms of *S. aureus* β-Lactamase

Ambler (1975) reported that *S. aureus* PC1 β-lactamase was readily digested without prior denaturation by a wide range of proteases. The enzyme can be purified easily in large amount (Richmond, 1963) and the primary (Ambler and Meadway, 1969; Ambler, 1975) and tertiary (Herzberg and Moult, 1987) structures have been elucidated. Furthermore, two mutant forms of *S. aureus* β-lactamase, which differ from wild-type by a single amino acid substitution (P54 mutant: Asp_{179} → Asn and P2 mutant: Thr_{71} → Ile, Ambler, 1979) and have about 5% of the specific activity of wild-type (Ambler, 1979), are even more susceptible to proteolysis than wild-type enzyme (Ambler, pers. comm.). For these reasons it was considered that wild-type and mutant forms of *S. aureus* β-lactamase would be an excellent choice of substrate to begin the investigation into the specificity of protease La.

Different β-lactamase producing strains of *S. aureus* release different proportions of their total enzyme into the medium (Novick and Richmond, 1965). Wild-type enzyme was purified from the constitutive strain PC1, which produces the highest proportion of extracellular enzyme, by the method of Richmond (1963) as modified by Ambler and Meadway (1969) (Table 2.8). The purity of the enzyme preparation was assessed by SDS-PAGE: limited contamination by proteins of molecular weights greater than 12,000, but much contamination by peptides, was observed (Figure 2.14). Ambler (1975) reported that some preparations of *S. aureus* β-lactamase contained unexpectedly high amounts of glycine after acid hydrolysis. Further investigation revealed that a peptide, of identical composition to the cell wall peptide (Gly_5, Ala_2, Glu, Lys), co-purified with *S. aureus* β-lactamase through ion-exchange and gel filtration chromatography. An amino acid analysis of the *S. aureus* PC1 β-lactamase preparation was performed by Professor R.P. Ambler, which indicated that the enzyme preparation may have been contaminated by such a peptide, since it contained much more Gly than expected for the normal protein and slightly more Ala, Glu and Lys

(Table 2.9). Ambler (1975) found that the peptide could be removed by gel filtration in 50% formic acid, suggesting that the peptide was not covalently linked to the enzyme. After this treatment, which was monitored by SDS-PAGE (Figure 2.15), the enzyme preparation had an amino acid composition similar to *bona fide S. aureus* PC1 β-lactamase (Table 2.9). The specific activity of the enzyme was 16.4 ±0.8μmol benzylpenicillin hydrolysed h^{-1} μg^{-1} at pH 7.0 (mean of triplicate determinations ±S.D.), slightly less than the value reported by Ambler (1975). Treatment to remove contaminating peptides led to a decrease in the specific activity of the enzyme to 8.2 ±0.8μmol benzylpenicillin hydrolysed h^{-1} μg^{-1}, which was probably caused by mild, irreversible denaturation of the enzyme after gel filtration in 50% formic acid.

TABLE 2.8 Purification of *S. aureus* PC1 β-Lactamase

PURIFICATION STEP	TOTAL ACTIVITY RECOVERED [a]	PERCENTAGE RECOVERY	
		STEP	OVERALL
Culture supernatant	4.2×10^6	-	-
Adsorption to phosphocellulose	3.8×10^6	90	90
Elution from phosphocellulose	1.8×10^6	47	42
Gel filtration chromatography	1.1×10^6	61	26
Dialysis	9.0×10^5	82	21
Lyophilization	6.0×10^5	67	14

a. μmol benzylpenicillin hydrolysed h^{-1}.

Using an identical protocol to the one employed for the PC1 form of the enzyme, purification of β-lactamase from *S. aureus* strain P54 was attempted. Purification relied upon the P54 form of the enzyme (of low specific activity) behaving in an analogous manner to the PC1 form, because the sensitivity of assays using benzylpenicillin was insufficient to detect activity in fractions eluted from columns (activity was barely discernible in highly concentrated fractions eluted from the phosphocellulose column). Material after lyophilization was analysed by SDS-PAGE which showed that the P54 form of the enzyme was purified successfully (Figure 2.16). The amino acid composition of the P54 enzyme preparation was similar to *bona fide S. aureus* PC1 β-lactamase (Table 2.10) and the specific activity of P54 enzyme was 1.2μmol benzylpenicillin hydrolysed h^{-1} μg^{-1}, about 7% of that of the PC1 enzyme.

FIGURE 2.14 Purity of *S. aureus* PC1 β-Lactamase Preparation

The enzyme was eluted from the phosphocellulose column in a large volume (about 200ml); and since the maximum volume that could be loaded onto the gel filtration column was only 40ml, five separate runs were required. After dialysis and lyophilization, a sample from each run (about 7μg) was analysed by SDS-PAGE using a 5-20% gradient gel, which was stained with Coomassie Blue.

Lane 1: about 30μg Run 1.
Lanes 2 & 8: BSA, 67,000; β-lactamase, 28,000; β-lactoglobulin, 18,000; cytochrome c, 12,000.
Lanes 3-7: Runs 1-5, respectively.

1 About 5mg S. aureus PC1 β-lactamase in 50% formic acid was loaded onto a Sephadex G-25 column (60cm x 1.5cm) and the A_{260} of the eluent is indicated.

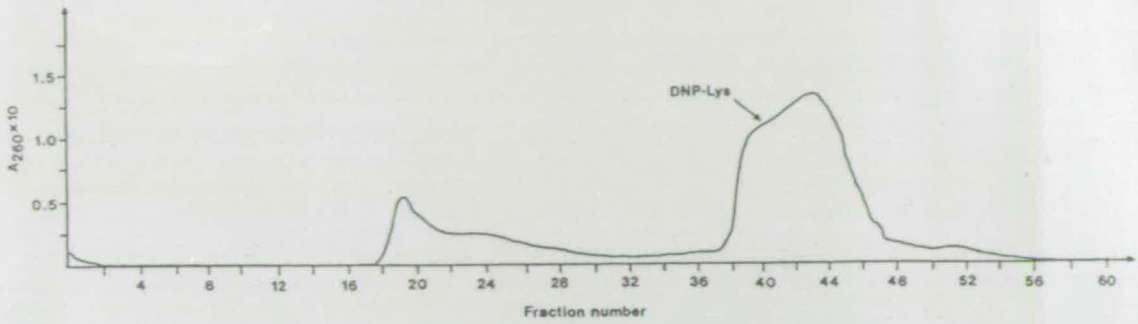
2 Fractions were analysed by SDS-PAGE using a 15% gel, which was stained with Coomassie Blue.

Lanes 1 & 14: BSA, 67,000; β-lactamase, 28,000; β-lactoglobulin, 18,000; cytochrome c, 12,000.

Lane 2: 100µl fraction 18.
Lane 3: 15µl fraction 19.
Lane 4: 15µl fraction 20.
Lane 5: 15µl fraction 21.
Lane 6: 100µl pooled fractions 23-26.
Lane 7: 100µl pooled fractions 27-30.
Lane 8: 100µl pooled fractions 35-37.
Lane 9: 100µl pooled fractions 38-39.
Lane 10: 100µl pooled fractions 40-43.
Lane 11: 100µl pooled fractions 44-47.
Lane 12: 100µl pooled fractions 48-55.
Lane 13: 20µg insulin.

FIGURE 2.15 Treatment to Remove Contaminating Peptides from *S. aureus* PC1 β-Lactamase

1 Elution Profile of Gel Filtration Column



2 SDS-PAGE of Fractions From the Gel Filtration Column

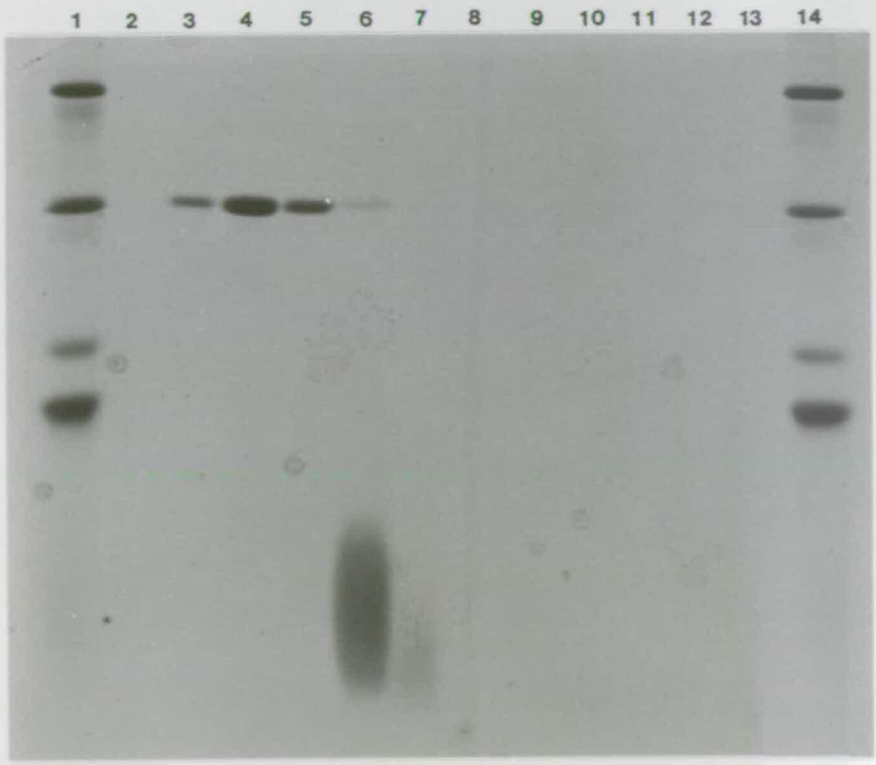


TABLE 2.9 Amino Acid Composition of *S. aureus* PC1 β-Lactamase

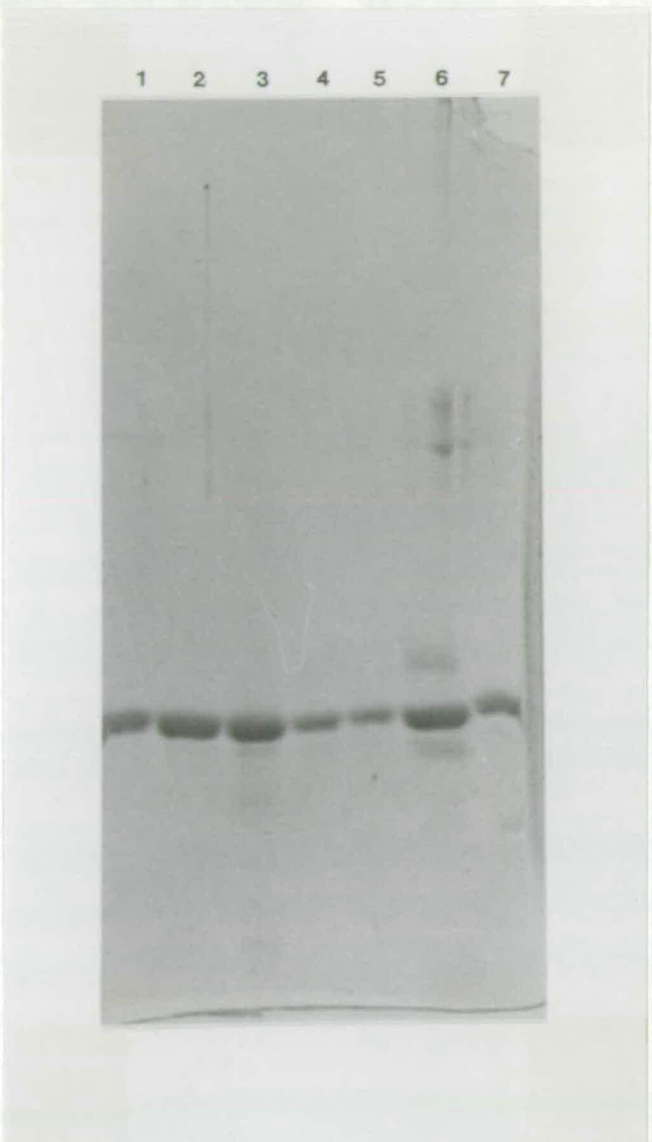
AMINO ACID	AMOUNT RECOVERED (μmol) [a]		RESIDUES MOLECULE^{-1} [b]		
	BEFORE	AFTER	BEFORE	AFTER	FROM SEQUENCE [c]
Lys	0.26	0.11	73	45	43
His	0.018	0.0049	5.1	2.0	2
Arg	0.047	0.0081	13	3.3	4
Cys	0.00	0.00	0.0	0.0	0
Asp	0.14	0.093	39	38	39
Thr	0.056	0.033	16	13	13
Ser	0.082	0.046	23	19	19
Glu	0.098	0.043	28	18	14
Pro	0.040	0.018	11	7.3	9
Gly	0.11	0.031	31	13	12
Ala	0.096	0.045	27	18	18
Val	0.057	0.038	16	15	16
Met	0.0097	0.0055	2.7	2.2	3
Ile	0.059	0.040	17	16	19
Leu	0.078	0.054	22	22	22
Tyr	0.038	0.028	11	11	11
Phe	0.024	0.014	6.8	5.7	7

a. The amino acid composition of the enzyme preparation, before (0.23mg protein) and after (0.16mg protein) gel filtration chromatography in 50% formic acid, was determined.

b. Residues molecule^{-1} were calculated relative to leucine, which was defined as 22.

c. Ambler (1975)

FIGURE 2.16 Purity of *S. aureus* P54 β-Lactamase Preparation



After lyophilization the preparation was analysed by SDS-PAGE using a 10% gel, which was stained with Coomassie Blue.

Lanes 1 & 7: 5µg S. aureus PC1 β-lactamase preparation.
Lanes 2 & 6: 10µg S. aureus PC1 β-lactamase preparation.
Lanes 3-5: 10µg, 3µg, and 1µg S. aureus P54 β-lactamase preparation, respectively.

TABLE 2.10 Amino Acid Composition of *S. aureus* P54 β-Lactamase

AMINO ACID	AMOUNT RECOVERED (μmol) [a]	RESIDUES MOLECULE^{-1} [b]	
		P54	PC1 (from sequence) [c]
Lys	0.062	55	43
His	0.0029	2.6	2
Arg	0.058	5.1	4
Cys	0.00	0.0	0
Asp	0.040	35	39
Thr	0.012	11	13
Ser	0.020	18	19
Glu	0.020	18	14
Pro	0.0084	7.4	9
Gly	0.018	16	12
Ala	0.020	18	18
Val	0.018	16	16
Met	0.0028	2.5	3
Ile	0.018	16	19
Leu	0.025	22	22
Tyr	0.013	11	12
Phe	0.0096	8.4	7

a. About 0.07mg P54 was used for analysis.

b. Residues molecule^{-1} were calculated relative to leucine, which was defined as 22.

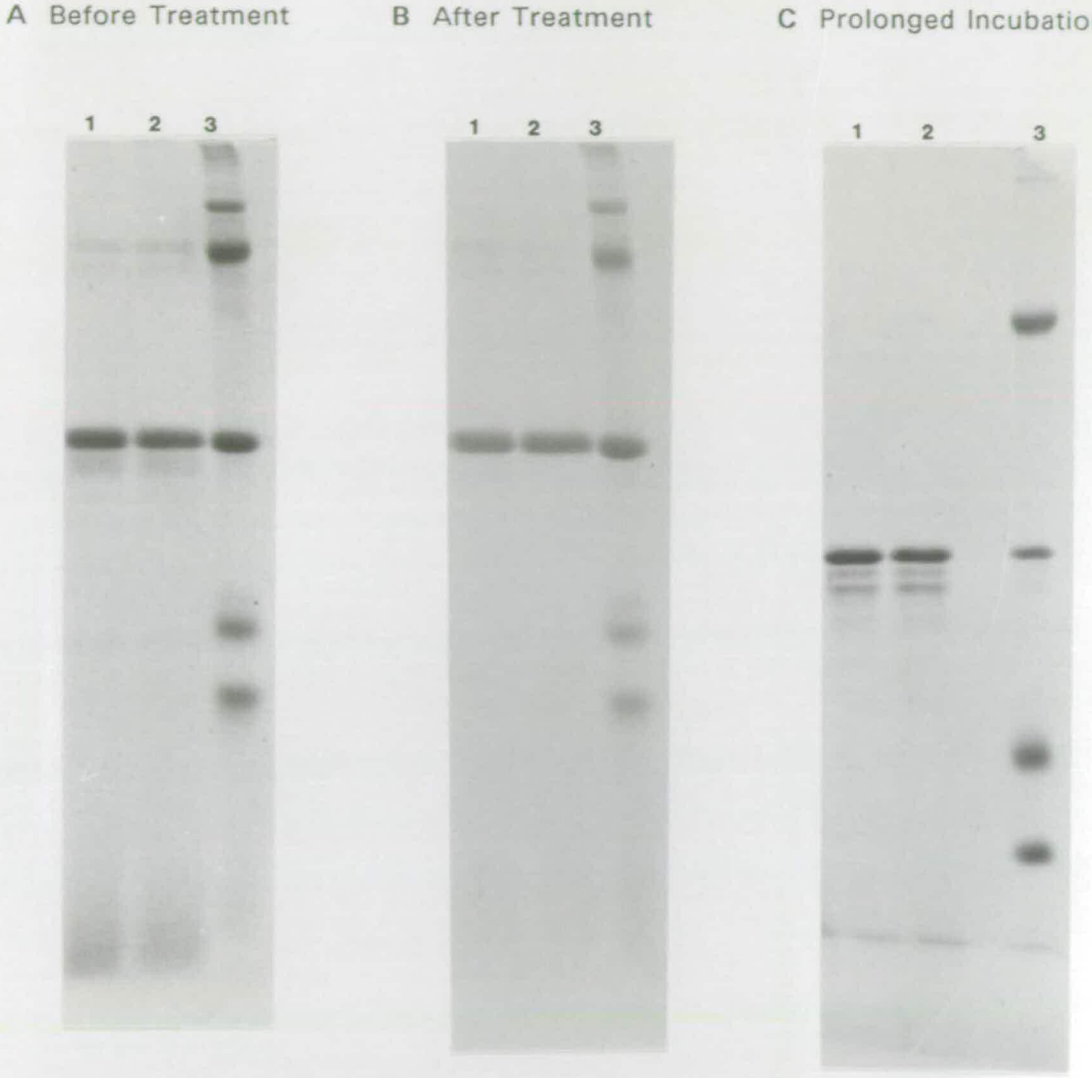
c. Ambler (1975)

2.2.3.2 Wild-Type and Mutant Forms of *S. aureus* β-Lactamase Are Not Degraded by Protease La Under Conditions Favourable for the Degradation of Radiolabelled Proteins

Mixtures containing wild-type *S. aureus* β-lactamase with, and without, protease La were analysed by SDS-PAGE (Figure 2.17). Degradation of *S. aureus* PC1 β-lactamase before, and after, treatment to remove contaminating (possibly cell wall) peptides was not detected: a difference in the intensity of the band corresponding to *S. aureus* PC1 β-lactamase in the mixtures with, and without, protease La was not observed; bands corresponding to degradation products were not seen in the mixture containing protease La. After prolonged incubation a difference in the intensity of the band corresponding to *S. aureus* PC1 β-lactamase in the mixtures with, and without, protease La was not apparent but, surprisingly, degradation products (molecular weights about 27,000 and 26,000) were seen in both mixtures (Figure 2.17). Digestion of *S. aureus* PC1 β-lactamase, in the absence of protease La, may have been caused by a protease in the β-lactamase preparation since several extracellular proteases have been purified from *S. aureus* (Arvidson, 1973; Arvidson *et al.*, 1973). Consequently, the *S. aureus* PC1 β-lactamase preparation was purified further by FPLC. Because *S. aureus* PC1 β-lactamase is a very basic protein (43 Lys residues), ion-exchange chromatography was performed using a cation-exchanger (Mono-S) at high pH (pH 8.7) (Figure 2.18). It was considered unlikely that a contaminating protease would adsorb to a cation-exchanger at such high pH since most proteins have isoelectric points less than 8.7.

Prior to FPLC, the purity of the *S. aureus* PC1 β-lactamase preparation had been assessed by SDS-PAGE, amino acid analysis and specific activity measurement, which had revealed negligible contamination by proteins but considerable contamination by peptides (Figure 2.14, Table 2.9 and Section 2.2.3.1). It was surprising, therefore, that when a sample of the *S. aureus* PC1 β-lactamase preparation was resolved by FPLC three large U.V. (280nm) absorbing peaks were detected, only one of which (peak III) hydrolysed nitrocefin (Figure 2.18). Peak III was comprised of *S. aureus* PC1 β-lactamase free from contamination by peptides (Figure 2.19.1). The nature of the material found in peaks I and II was not investigated further. Possibly, peak II consisted of degradation products of *S. aureus* PC1 β-lactamase formed during storage, because it seems unlikely that other proteins would adsorb to the column at this pH. The largest peak (peak I) probably contained the contaminating peptides (composed of Gly, Ala, Glu and basic amino acids, Table 2.9) although other U.V. absorbing material must have been present.

FIGURE 2.17 Degradation of *S. aureus* PC1 β-Lactamase by Protease La Was Not Observed



Reaction mixtures with and without protease La (enzyme subunit:protein molar ratio of 1:50), contained 30mM Tris/HCl pH 8.0, 15mM $MgCl_2$ and 3mM ATP, in a final volume of 30µl. After incubation for 90min (A & B) or 14h (C), the reaction mixtures were analysed by SDS-PAGE using a 12.5% gel, which was stained with Coomassie Blue. A: reaction mixtures contained about 3µg S. aureus PC1 β-lactamase before treatment to remove contaminating peptides; B & C: mixtures contained the same amount of enzyme, after treatment.

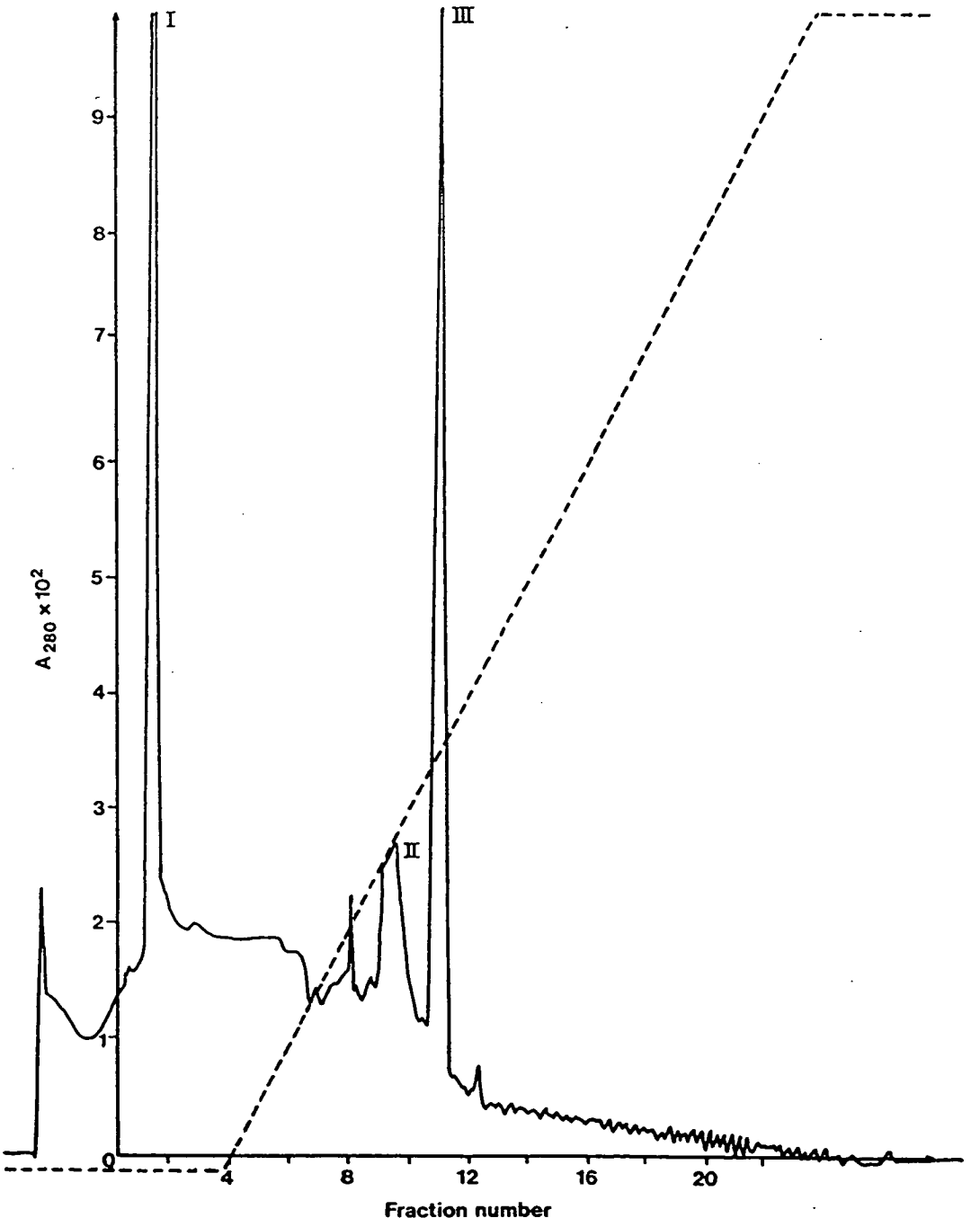
Lane 1 (A, B, & C): reaction mixtures – protease La.

Lane 2 (A, B, & C): reaction mixtures + protease La.

Lane 3 (A & B): phosphorylase b, 94,000; BSA, 67,000; β-lactamase, 28,000; β-lactoglobulin, 18,000; cytochrome c, 12,000.

Lane 3 (C): as above, but phosphorylase b omitted.

FIGURE 2.18 Purification of *S. aureus* PC1 β-Lactamase by FPLC



S. aureus PC1 β-lactamase (about 100μg) was loaded onto a Mono-S column equilibrated with 50mM BICINE pH 8.7 (Buffer A). The column was eluted with a salt gradient comprising Buffer A and Buffer B (50mM BICINE pH 8.7, 1.0M $CaCl_2$). The percentage of Buffer B in the gradient (------), and the A_{280} of the eluent (———), are indicated.

1 S. aureus PC1 β-lactamase after purification by FPLC was analysed by SDS-PAGE using a 10% gel, which was stained with Coomassie Blue.

Lane 1: phosphorylase b, 94,000; BSA, 67,000; β-lactamase, 28,000; β-lactoglobulin, 18,000; cytochrome c, 12,000.

Lane 2: about 5μg S. aureus PC1 β-lactamase.

---

2 For S. aureus P54 (lanes 1-4), P2 (lanes 5-8), and PC1 (lanes 9-12) β-lactamases, four reaction mixtures each were prepared containing: 50mM Tris/HCl pH 8.0, 25mM $MgCl_2$, and about 8μg, 4μg, or 2μg protein (P54, P2, & PC1, respectively), in a final volume of 30μl. ATP (final concentration 3mM) was added to two of the reaction mixtures, only one of which was incubated; ATP and protease La (0.3μg) were added to another; and protease La alone was added to the remaining mix. After incubation at 37°C for 5h, the reaction mixtures were analysed by SDS-PAGE in a 15% gel, which was stained with Coomassie Blue.

Lanes 1, 5, & 9: reaction mixtures + ATP, but not incubated.

Lanes 2, 6, & 10: reaction mixtures + ATP.

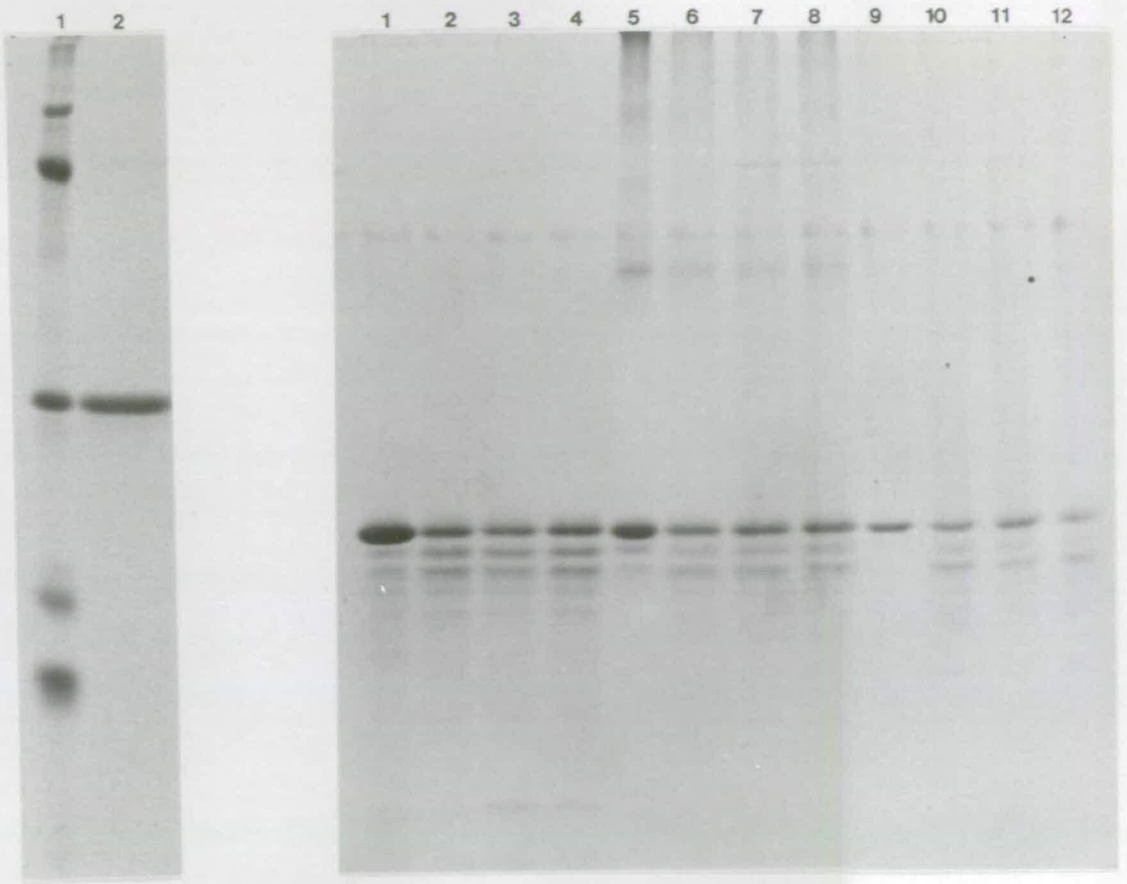
Lanes 3, 7, & 11: reaction mixtures + protease La & ATP.

Lanes 4, 8, & 12: reaction mixtures + protease La.

FIGURE 2.19 Degradation of Wild-Type and Mutant Forms of *S. aureus* β-Lactamase by Protease La Was Not Observed

1 FPLC Purified PC1 β-Lactamase

2 PC1, P2, & P54 β-Lactamases Incubated With Protease La



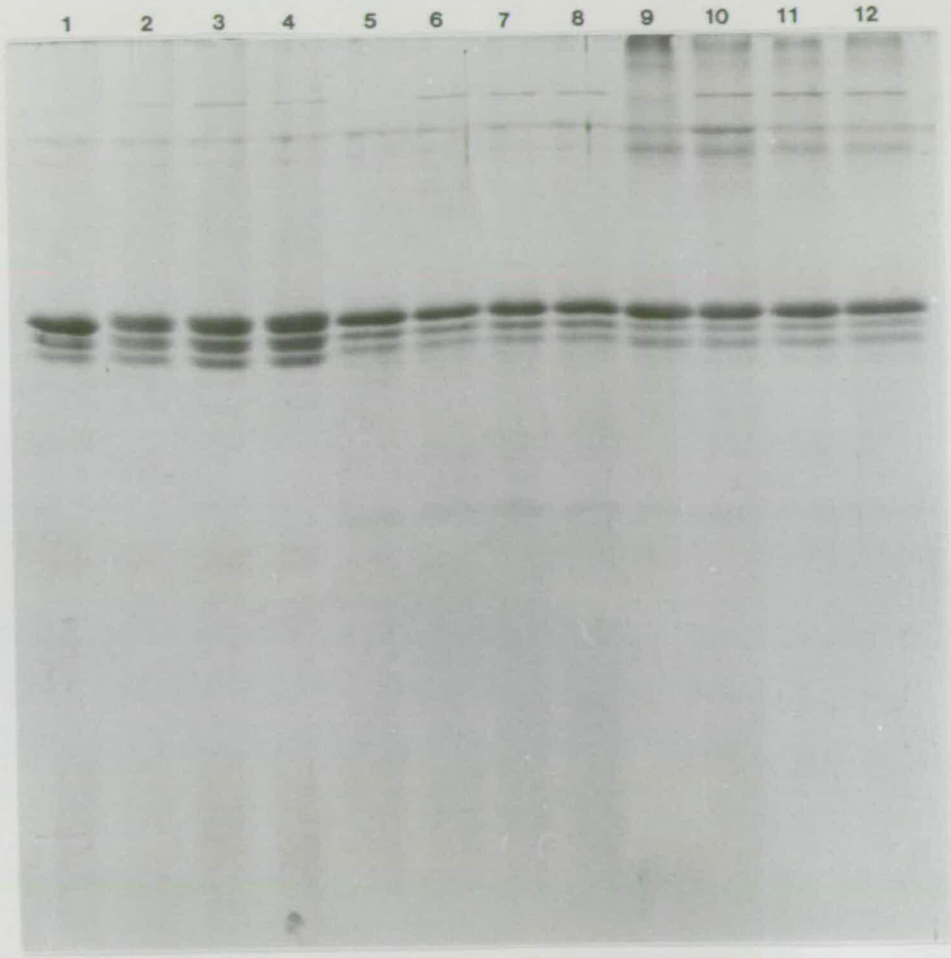
In accord with earlier findings, *S. aureus* PC1 β-lactamase purified by FPLC was not digested by protease La (Figure 2.19.2). However, degradation products were observed in the mixtures with, and without, protease La, despite purification by FPLC. These products may have been caused by auto-digestion (serine proteases and β-lactamases have several features in common; Herzberg and Moult, 1987) or, rather more plausibly, by contamination of the mixtures by proteases. Thus it appears that *S. aureus* PC1 β-lactamase is extremely susceptible to degradation by proteases other than protease La.

Mixtures containing the mutant forms of *S. aureus* β-lactamase with, and without, protease La were also analysed by SDS-PAGE (Figure 2.19.2). The P2 and P54 forms of the β-lactamase behaved in an analogous manner to the PC1 form: digestion was not detected but bands corresponding to degradation products (of the same molecular weights as products of PC1 β-lactamase) were observed in all the mixtures after prolonged incubation (Figure 2.19.2). Addition of single-stranded DNA to mixtures did not stimulate degradation of either wild-type or mutant forms of *S. aureus* β-lactamase by protease La (Figure 2.20). The failure of protease La to digest the P2 and P54 forms of *S. aureus* β-lactamase was unexpected. Protease La is known to digest abnormal proteins *in vivo*, and these mutant forms had been found to be extremely sensitive to degradation by trypsin: one thousand times less trypsin was required to digest the P2 and P54 forms of the enzyme (Figure 2.22) compared with the PC1 form (Figure 2.21).

2.2.3.3 Evidence That Protease La is a (Highly) Selective Protease

In order to find a substrate of protease La, fourteen readily available, well characterized protein preparations were incubated with the protease, and the mixtures were analysed by SDS-PAGE. Degradation of a protein would be revealed by the disappearance of the band corresponding to the protein on a polyacrylamide gel. By this method, complete digestion of only two of the protein preparations used (bovine α-lactalbumin and bovine casein) was detected; limited degradation of two protein preparations (horse heart cytochrome *c* and chick erythrocyte histone) was also detected (Figure 2.23). Heat denaturation did not increase the susceptibility of five protein preparations to degradation by protease La (Figure 2.24); although an increase was observed after more drastic denaturation of a β-lactoglobulin preparation by reductive methylation or by oxidation with performic acid (Figure 2.25). However, the extent of digestion of both the methyl- and oxidized β-lactoglobulin preparations was much less than that of the α-lactalbumin and casein preparations (Figure 2.23). These

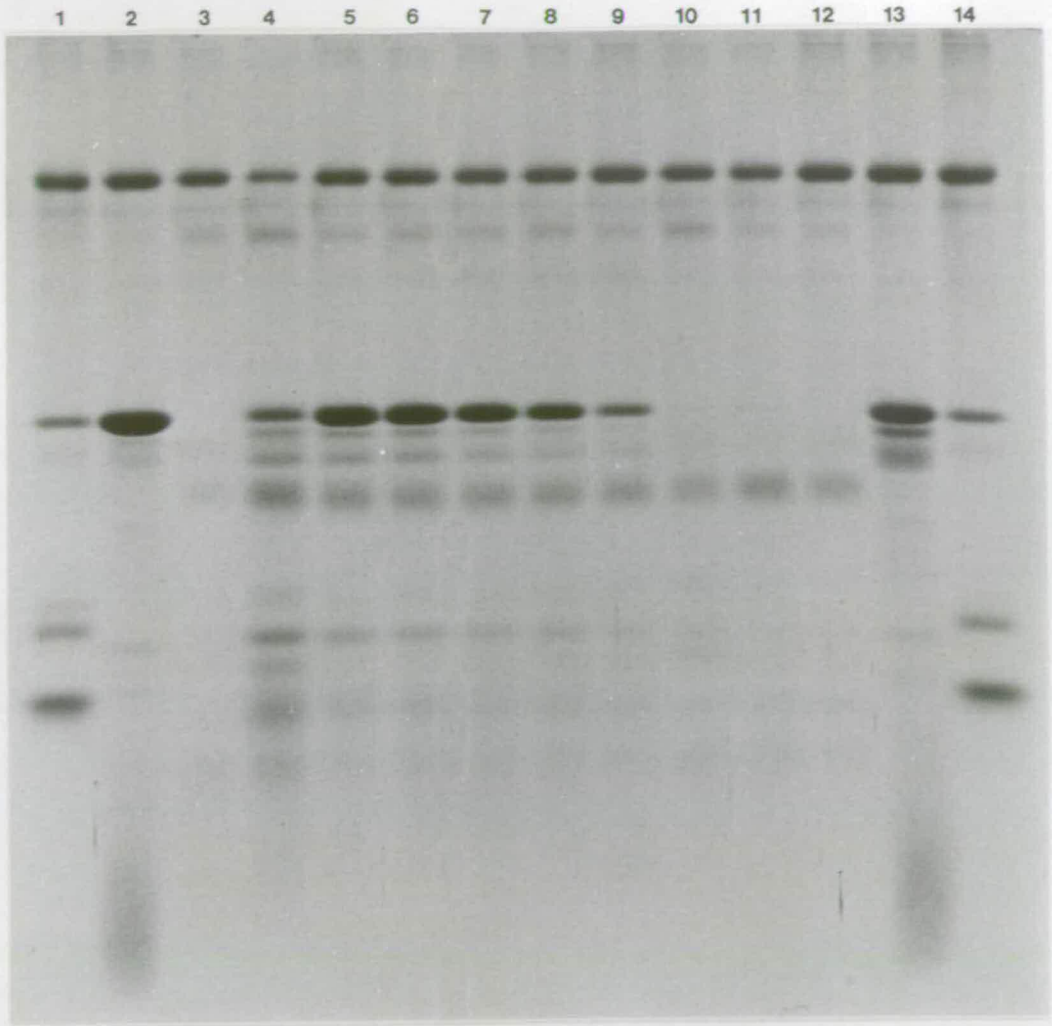
FIGURE 2.20 Degradation of Wild-Type and Mutant Forms of *S. aureus* β-Lactamase by Protease La in the Presence of DNA Was Not Observed



For S. aureus PC1 (lanes 1-4), P54 (lanes 5-8) and P2 (lanes 9-12) β-lactamases, four reaction mixtures containing 50mM Tris/HCl pH 8.0, 25mM $MgCl_2$, 3mM ATP, and about 5μg protein, in a final volume of 30μl, were prepared. No addition was made to one reaction mixture (lanes 1, 5, & 9); protease La (enzyme subunit:protein molar ratio of 1:40) was added to another (lanes 2, 6, & 10); protease La and ssDNA (final concentration 0.1 or 10μg ml^{-1}) were added to the remaining mixes. After incubation at 37°C for 16h, the reaction mixtures were analysed by SDS-PAGE using a 15% gel, which was stained with Coomassie Blue.

Lanes 1, 5, & 9: reaction mixtures, no addition.
Lanes 2, 6, & 10: reaction mixtures + protease La.
Lanes 3, 7, & 11: reaction mixtures + protease La & ssDNA (0.1μg ml^{-1}).
Lanes 4, 8, & 12: reaction mixtures + protease La & ssDNA (10μg ml^{-1}).

FIGURE 2.21 Susceptibility of Wild-Type *S. aureus* β-Lactamase to Proteolysis



S. aureus PC1 β-lactamase before treatment to remove contaminating peptides (about 80μg) was incubated at 37°C with 80μg trypsin in buffer containing 20mM Tris/HCl pH 7.6, and 20mM NaCl, in a final volume of 80μl (Sigal et al., 1984). At various times, 10μl aliquots were removed and analysed by SDS-PAGE using a 10-15% gradient gel, which was stained with Coomassie Blue. Prior to loading the gel, BSA was added to the samples as an internal standard.

Lanes 1 & 14: BSA, 67,000; β-lactamase, 28,000; β-lactoglobulin, 18,000; cytochrome c, 12,000.

Lane 2: 10μg S. aureus PC1 β-lactamase.

Lane 3: 10μg trypsin.

Lanes 4-11: 0.5min, 5min, 10min, 15min, 20min, 30min, 60min, & 120min incubations, respectively.

Lane 12: 10μg trypsin, incubated 120min.

Lane 13: 10μg S. aureus PC1 β-lactamase, incubated 120min.

1 S. aureus P2 β-lactamase (about 100μg) was incubated at 37°C with either 100μg (A) or 100ng (B) trypsin in buffer containing 20mM Tris/HCl pH 7.6, and 20mM NaCl, in a final volume of 100μl. At various times, 10μl aliquots were removed and analysed by SDS-PAGE using a 15% gel, which was stained with Coomassie Blue. Prior to loading the gel, BSA was added to the samples as an internal standard. For both A & B:

Lanes 1 & 14: BSA, 67,000; carbonic anhydrase, 29,000; β-lactoglobulin, 18,000; cytochrome c, 12,000.

Lane 2: 10μg and 10ng trypsin, respectively.

Lane 3: 10μg S. aureus P2 β-lactamase.

Lanes 4-11: 0.5min, 5min, 10min, 15min, 20min, 30min, 60min, & 120min incubations, respectively.

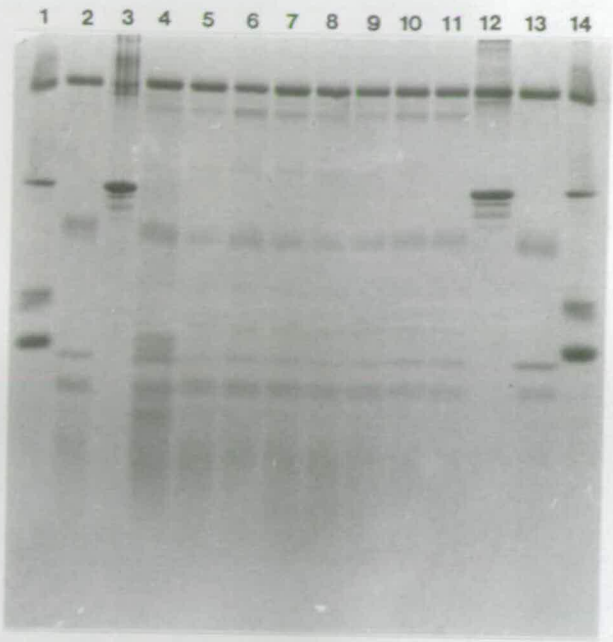
Lane 12: 10μg S. aureus P2 β-lactamase, incubated 120min.

Lane 13: 10μg and 10ng trypsin, incubated 120min, respectively.

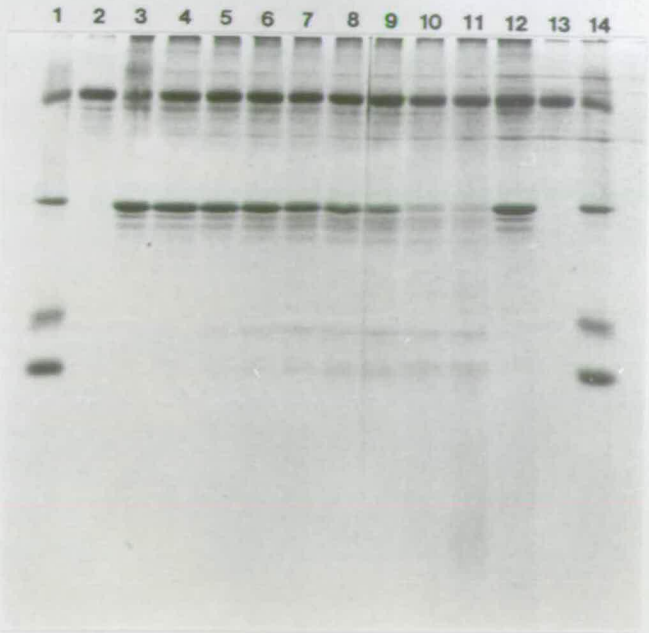
FIGURE 2.22 Susceptibility of Mutant Forms of *S. aureus* β-Lactamase to Proteolysis

1 P2 Form

A



B



2 S. aureus P54 β-lactamase (about 80μg) was incubated at 37°C with either 80μg (A) or 80ng (B) trypsin in buffer containing 20mM Tris/HCl pH 7.6, and 20mM NaCl, in a final volume of 80μl. At various times, 10μl aliquots were removed and analysed by SDS-PAGE using a 10-15% gradient gel, which was stained with Coomassie Blue. Prior to loading the gel, BSA was added to the samples as an internal standard.

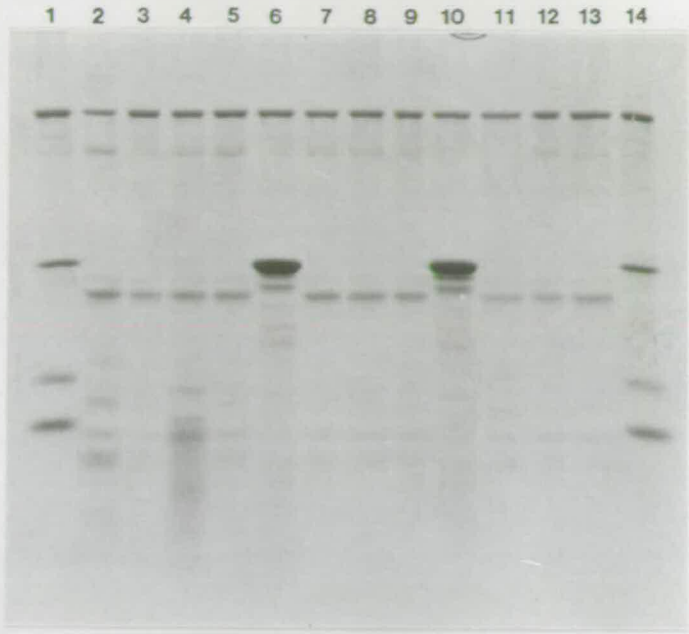
A Lanes 1 & 14: BSA, 67,000; β-lactamase, 28,000; β-lactoglobulin, 18,000; cytochrome c, 12,000.
Lane 2: 20μg trypsin.
Lane 3: 10μg trypsin, incubated 120min.
Lanes 4-5, 7-9, & 11-13: 0.5min, 5min, 10min, 15min, 20min, 30min, 60min, & 120min incubations, respectively.
Lane 6: 10μg S. aureus P54 β-lactamase.
Lane 10: 10μg S. aureus P54 β-lactamase, incubated 120min.

B Lanes 1 & 13: BSA, 67,000; β-lactamase, 28,000; β-lactoglobulin, 18,000; cytochrome c, 12,000.
Lane 2: 20μg trypsin.
Lane 3: 10μg trypsin, incubated 120min.
Lanes 4-5, 7-9, & 11-12: 0.5min, 5min, 10min, 15min, 20min, 30min, & 60min incubations, respectively.
Lane 6: 10μg S. aureus P54 β-lactamase.
Lane 10: 10μg S. aureus P54 β-lactamase, incubated 120min.

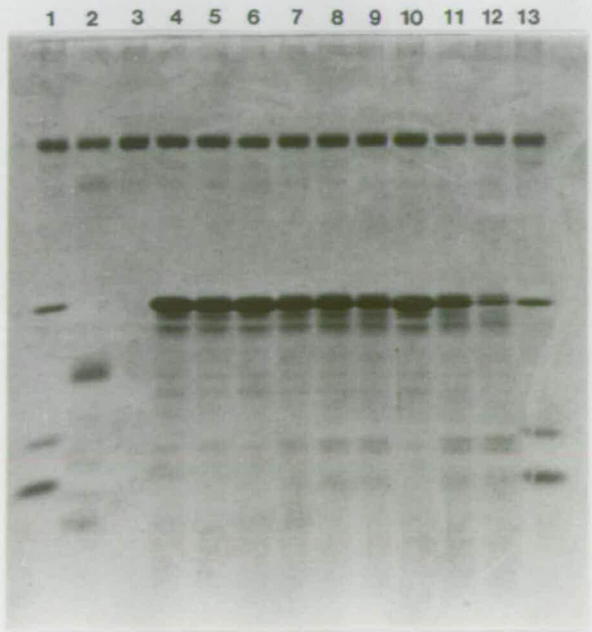
FIGURE 2.22 (continued)

2 P54 Form

A



B



For each protein, four reaction mixtures were prepared containing 50mM Tris/HCl pH 8.0, 25mM $MgCl_2$, 3mM ATP, and about 5μg protein, in a final volume of 30μl. In addition, the four reaction mixtures contained:

Reaction 1: no further additions (lanes 1, 5, & 9).

Reaction 2: protease La (enzyme subunit:protein molar ratio 1:40) (lanes 2, 6, & 10).

Reaction 3: protease La & ssDNA (0.1μg ml^{-1}) (lanes 3, 7, & 11).

Reaction 4: protease La & ssDNA (10μg ml^{-1}) (lanes 4, 8, & 12).

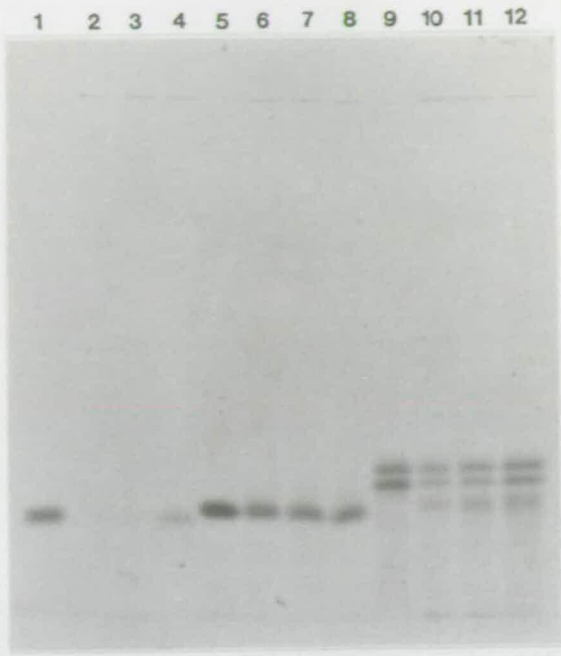
After incubation at 37°C for 15h, the reaction mixtures were analysed by SDS-PAGE using a 17.5% gel:

A Lanes 1-4: bovine α-lactalbumin.
Lanes 5-8: horse heart cytochrome c.
Lanes 9-12: chick erythrocyte histone.

B Lanes 1-4: bovine pancreatic RNase.
Lanes 5-8: hen egg white lysozyme.
Lanes 9-12: bovine casein.

FIGURE 2.23 Degradation of Native Proteins by Protease La

A



B

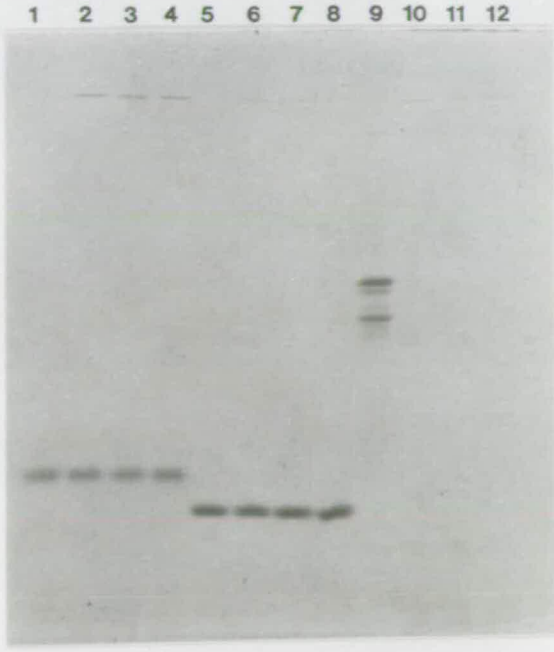
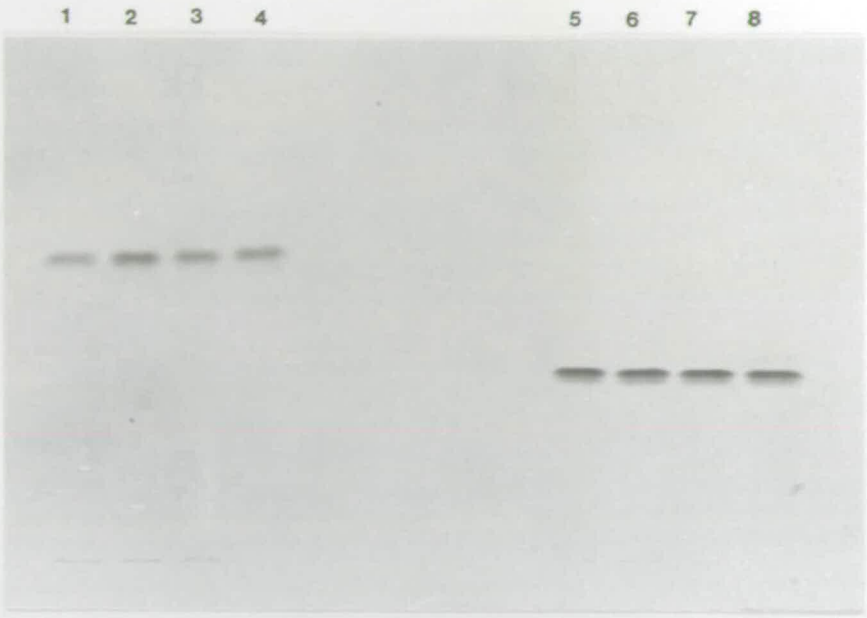
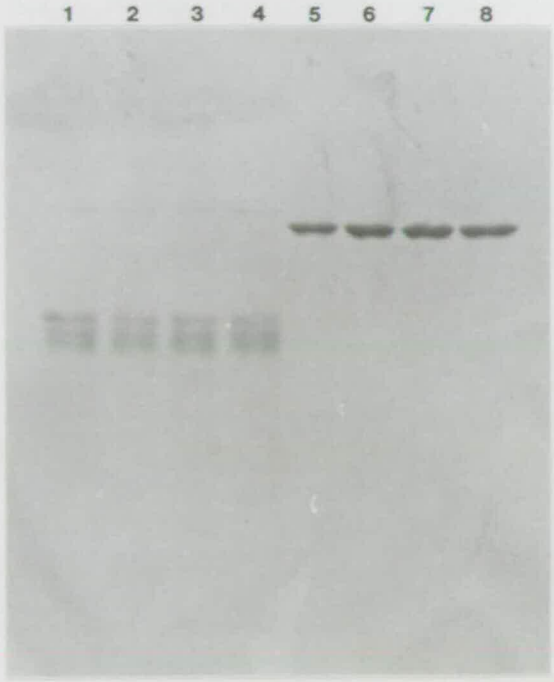


FIGURE 2.23 (continued)

C



D



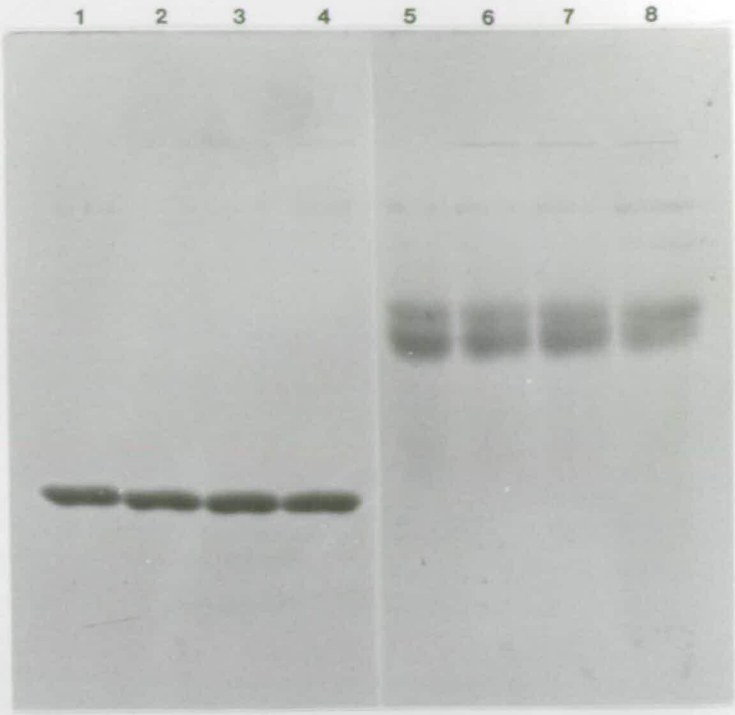
The experiment was performed as already described, except that the reaction mixtures were analysed using either a 15% gel (C) or a 12.5% gel (D):

C Lanes 1–4: E. coli RTEM β-lactamase.
Lanes 5–8: soybean trypsin inhibitor.

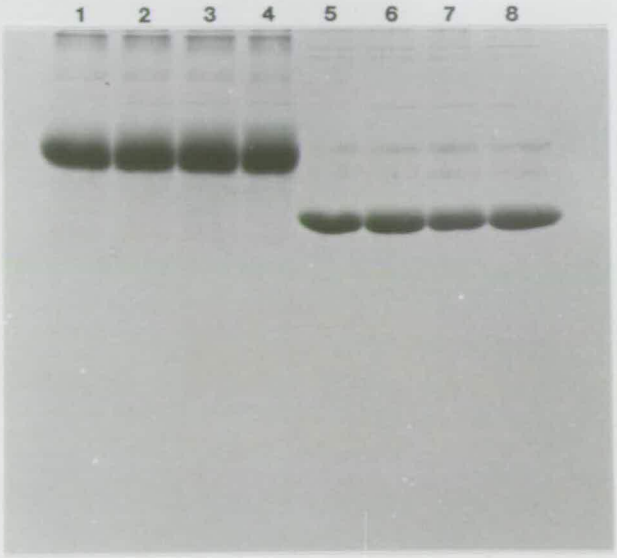
D Lanes 1–4: rabbit α-glycerophosphate dehydrogenase.
Lanes 5–8: yeast enolase.

FIGURE 2.23 (continued)

E



F



The experiment was performed as already described, except that the reaction mixtures were analysed using 12.5% gels:

E Lanes 1–4: bovine erythrocyte carbonic anhydrase.
Lanes 5–8: hen egg albumin.

F Lanes 1–4: bovine serum albumin.
Lanes 5–8: yeast 3-phosphoglycerate phosphokinase.

Proteins (about 5μg) were incubated at 37°C for 19h either with (lanes 1, 3, 5, 7, 9, & 11), or without (lanes 2, 4, 6, 8, 10, & 12), protease La (enzyme subunit:protein molar ratio of 1:80) in buffer containing 25mM Tris/HCl pH 8.0, 12mM $MgCl_2$, and 3mM ATP, in a final volume of 20μl. After incubation, the reaction mixtures were analysed by SDS-PAGE using a 12.5% gel, which was stained with Coomassie Blue. The proteins used were either in the native state (A), or denatured by boiling for 2min and chilling in dry ice (B). The protein substrates used were (for both A & B):

Lanes 1 & 2: bovine α-lactalbumin.

Lanes 3 & 4: soybean trypsin inhibitor.

Lanes 5 & 6: bovine erythrocyte carbonic anhydrase.

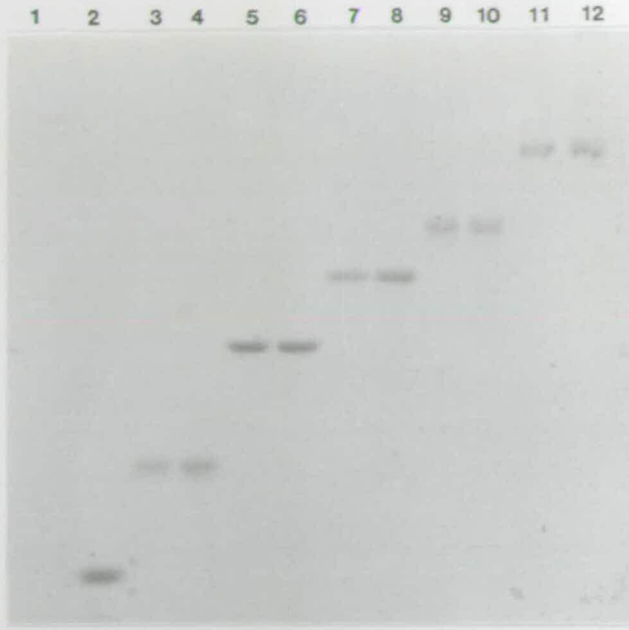
Lanes 7 & 8: rabbit glyceraldehyde-3-phosphate dehydrogenase.

Lanes 9 & 10: hen egg albumin.

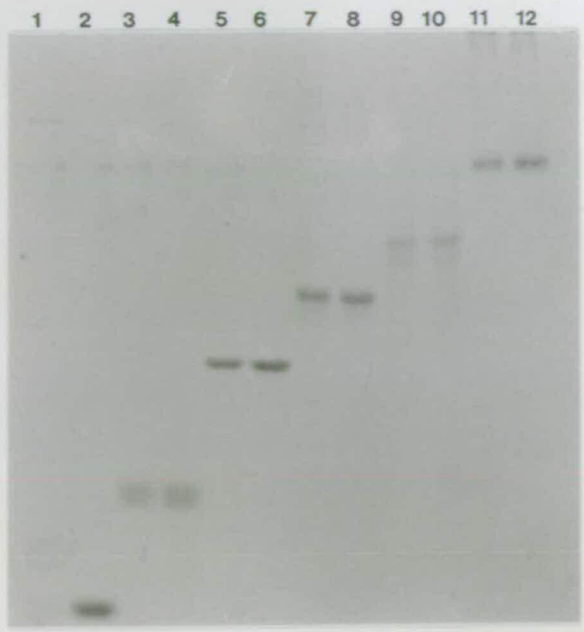
Lanes 11 & 12: bovine serum albumin.

FIGURE 2.24 Effect of Heat Denaturation on the Degradation of Proteins by Protease La

A Native Proteins



B Denatured Proteins



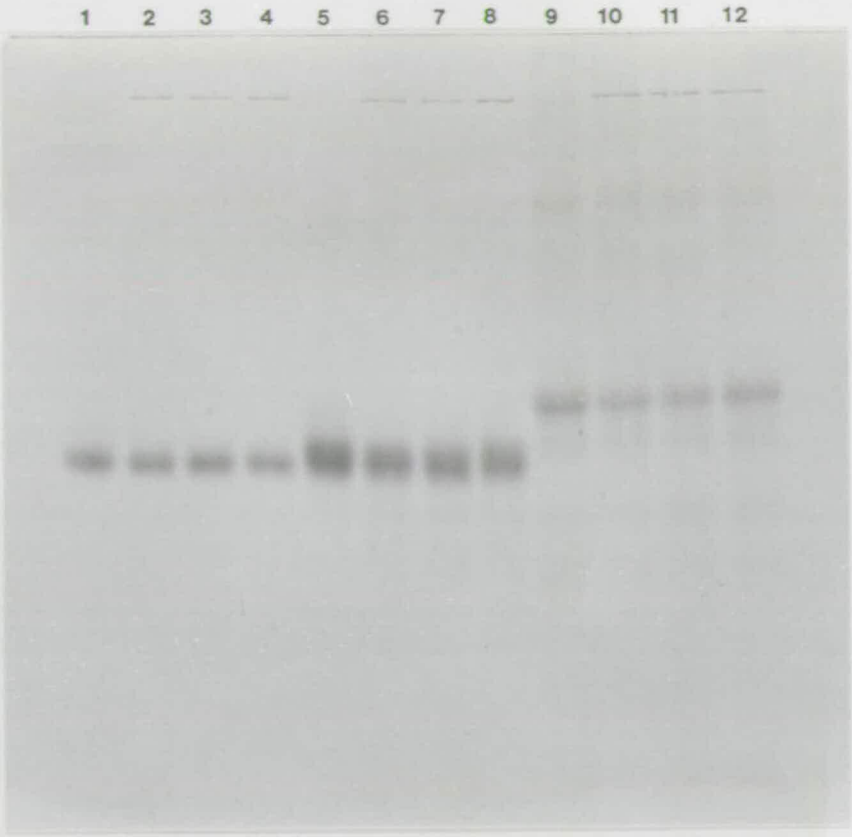
results indicate that protease La is (highly) selective in degrading native proteins, and that denaturation by heat, reductive methylation or oxidation is not sufficient to convert a protein into a good substrate. Interestingly, single-stranded DNA inhibited degradation of the α-lactalbumin, histone and oxidized β-lactoglobulin preparations by protease La, especially at high concentrations, but had no discernible effect on the digestion of cytochrome *c* or methyl-β-lactoglobulin preparations (Figures 2.23 and 2.25). Since casein was totally digested in the presence and absence of DNA, the effect of DNA could not be determined (Figure 2.23).

In contrast to the α-lactalbumin preparation, the preparation of casein was heterogeneous (Figure 2.23) and so further studies on protease La were carried out using α-lactalbumin. A molar ratio of protease La subunit to α-lactalbumin of more than 1:80 was required for complete digestion of α-lactalbumin in 15h (Figure 2.26.1). A degradation product (molecular weight about 10,000) was observed in all the reaction mixtures which contained protease La, whether or not ATP was present (Figure 2.26.1). Similarly, a degradation product was detected when [^{14}C]-α-lactalbumin was incubated with protease La (Figure 2.11.2). Degradation of α-lactalbumin by protease La, in the absence of ATP, may have been caused by a protease in the α-lactalbumin preparation, but this seems unlikely because degradation products were not observed when α-lactalbumin was incubated without protease La. However, it is possible that a factor present in the protease La preparation stimulates a protease present in the α-lactalbumin preparation. Alternatively the protease La preparation may have been contaminated by a protease but, as discussed earlier (Section 2.2.2), there are other possible explanations:

1. The proteolytic activity of protease La is stimulated by and not dependent upon ATP.
2. The α-lactalbumin preparation contains a factor (possibly ATP) which supports limited degradation by protease La.

Evidence to support explanation 1 is given by the finding that ATP prevents heat inactivation of protease La (Larimore *et al.*, 1982) together with the observation that the band on the polyacrylamide gel corresponding to protease La was more intense in reaction mixtures which contained ATP, than in those without ATP (Figure 2.26.1). These results suggest that ATP prevents protease La from undergoing ATP-independent auto-digestion. Further evidence was provided when α-lactalbumin was incubated with individual, active fractions from the final step in the purification of protease La (gel filtration using Sephacryl S-300): all the fractions degraded

FIGURE 2.25 Effect of Denaturation on the Degradation of β-Lactoglobulin By Protease La



For both native and denatured bovine β-lactoglobulin, four reaction mixtures were prepared containing 50mM Tris/HCl pH 8.0, 25mM $MgCl_2$, 3mM ATP, and about 5μg protein, in a final volume of 30μl. In addition, the four reaction mixtures contained:

Reaction 1: no additions (lanes 1, 5, & 9).

Reaction 2: + protease La (enzyme subunit:protein molar ratio 1:40) (lanes 2, 6, & 10).

Reaction 3: + protease La & ssDNA (0.1μg ml^{-1}) (lanes 3, 7, & 11).

Reaction 4: + protease La & ssDNA (10μg ml^{-1}) (lanes 4, 8, & 12).

After incubation at 37°C for 15h, the reaction mixtures were analysed by SDS-PAGE using a 15% gel:

Lanes 1-4: native β-lactoglobulin.

Lanes 5-8: methyl-β-lactoglobulin.

Lanes 9-12: oxidized β-lactoglobulin.

1 Reaction mixtures with, and without, ATP (final concentration 3mM) contained: 30mM Tris/HCl pH 8.0, 15mM $MgCl_2$, about 5μg α-lactalbumin, and varying amounts of protease La, in a final volume of 30μl. After incubation at 37°C for 15h, the mixtures were analysed by SDS-PAGE using a 17.5% gel, which was stained with Coomassie Blue:

Lanes 1 & 16: BSA, 67,000; β-lactamase, 28,000; β-lactoglobulin, 18,000; cytochrome c, 12,000.

Lane 2: + ATP only.

Lane 3: + protease La (enzyme subunit:α-lactalbumin molar ratio of 1:40) & ATP.

Lane 4: + protease La (molar ratio as in 3) only.

Lanes 5, 7, 9, 11, & 13: + protease La (molar ratios of 1:20, 1:40, 1:80, 1:160, & 1:320, respectively) & ATP.

Lanes 6, 8, 10, 12, & 14: + protease La (molar ratios as above), - ATP.

Lane 15: - ATP.

2 Reaction mixtures with, and without, ATP (final concentration 3mM) contained: 50mM Tris/HCl, pH 8.0, 25mM $MgCl_2$, about 5μg α-lactalbumin, and appropriate amounts of the fractions from the Sephacryl-S300 column containing protease La, in a final volume of 30μl. After incubation at 37°C for 14h, the mixtures were analysed by SDS-PAGE using a 15% gel, which was stained with Coomassie Blue:

Lane 1: BSA, 67,000; β-lactamase, 28,000; β-lactoglobulin, 18,000; cytochrome c, 12,000.

Lane 2: + ATP.

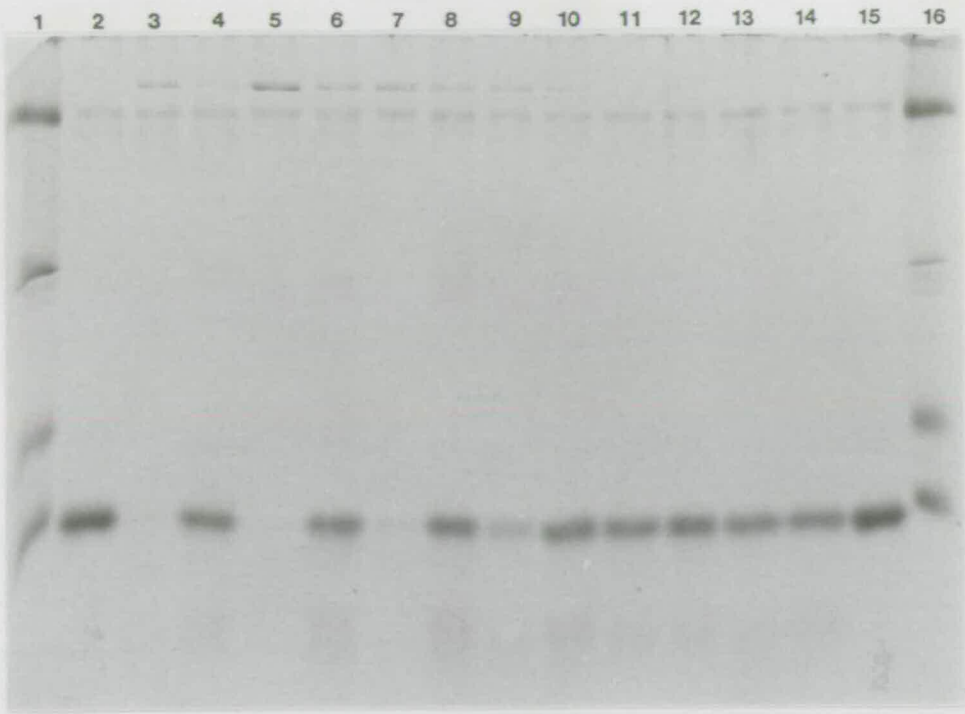
Lanes 3, 5, 7, 9, 11, & 13: + fractions 14, 15, 16, 17, 18, & 19, respectively, + ATP.

Lanes 4, 6, 8, 10, 12, & 14: + fractions 14, 15, 16, 17, 18, & 19, respectively, - ATP.

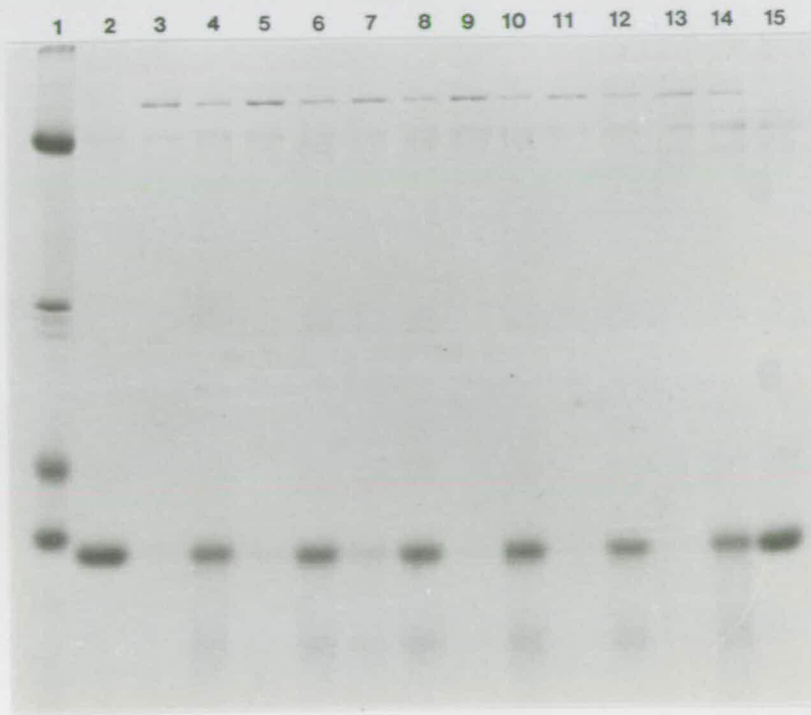
Lane 15: - ATP.

FIGURE 2.26 Degradation of α-Lactalbumin By Protease La

1 With Varying Amounts of Protease La



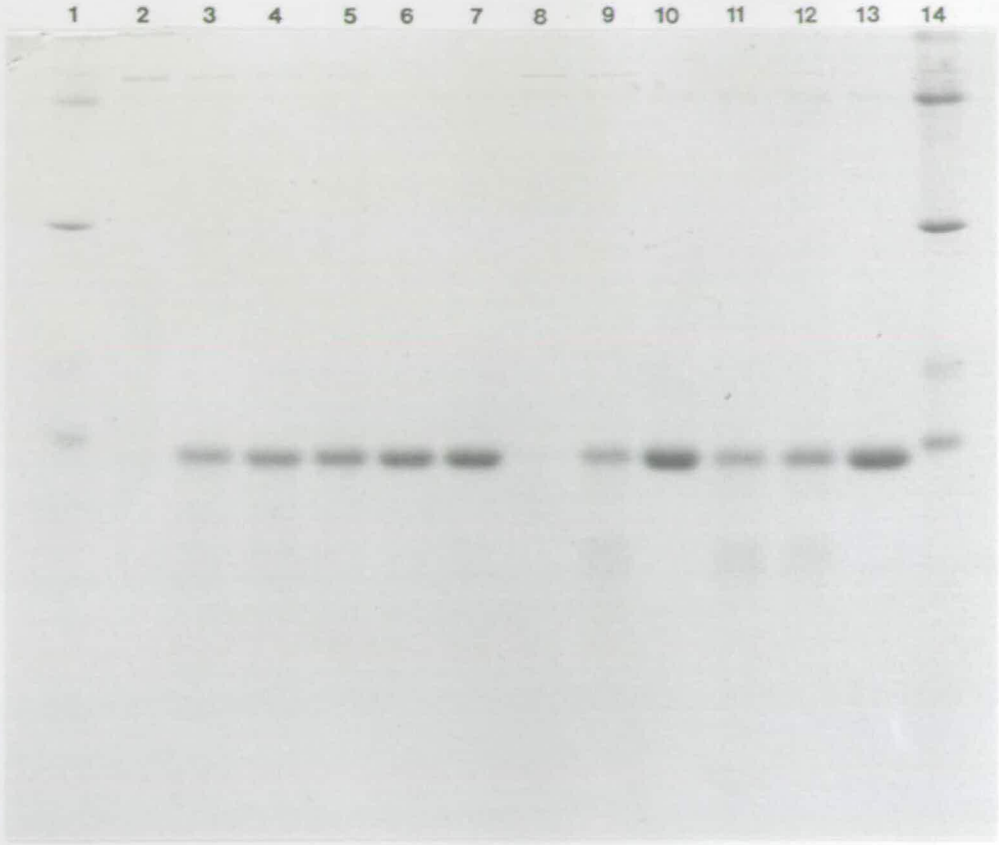
2 With Sephacryl-S300 Fractions



α-lactalbumin in the absence of ATP and, once again, a greater amount of protease La was detected in reaction mixtures that contained ATP, than in those without ATP (Figure 2.26.2). However, two points must be borne in mind: first, intracellular proteases are large proteins (Goldberg *et al.*, 1982) which may have co-purified with protease La through ion-exchange chromatography and gel filtration and second, protease La incubated in the absence of ATP may become a substrate of a contaminating protease. Surprisingly, when a different preparation of α-lactalbumin was incubated with protease La (no ATP), no degradation product was detected even though one had been observed with the original preparation under these conditions (Figure 2.27). Possibly, some preparations of α-lactalbumin contain a factor (perhaps ATP) which supports limited digestion by protease La in the absence of exogenous ATP; or alternatively, perhaps some preparations are more susceptible to degradation by either a contaminating protease or the putative ATP-independent proteolytic activity of protease La. Another explanation could be that some preparations of α-lactalbumin contain a protease which is stimulated by a factor in the protease La preparation.

Degradation of α-lactalbumin by protease La in the presence of ATP increased with length of incubation up to 8h, when digestion was complete (Figure 2.28.1). After brief incubation of α-lactalbumin and protease La in the absence of ATP, neither degradation of α-lactalbumin nor loss of protease La was detected, whereas after prolonged incubation digestion was detected (a faint band corresponding to a degradation product was observed) and so was loss of protease La (Figure 2.28.1). A high concentration of ATP (K_m 27μM; Waxman and Goldberg, 1982) was required for digestion of α-lactalbumin by protease La; negligible degradation had occurred after incubation for 6h with 0.1mM ATP (Figure 2.28.2). A possible explanation for this finding is that the ATP was contaminated by ADP, AMP and phosphate which inhibit protease La (Larimore *et al.*, 1982). Omission of Mg^{2+} ions greatly decreased degradation of α-lactalbumin by protease La and, as before, addition of single-stranded DNA inhibited digestion of α-lactalbumin by protease La (Figure 2.28.2).

FIGURE 2.27 Degradation of Different Preparations of α-Lactalbumin By Protease La



For each preparation of α-lactalbumin, six reaction mixtures were prepared containing 30mM Tris/HCl pH 8.0, 15mM $MgCl_2$, and about 5μg α-lactalbumin, in a final volume of 30μl. In addition, the six reaction mixtures contained:

Reaction 1: + protease La (enzyme subunit:α-lactalbumin molar ratio of 1:80) & ATP (final concentration 3mM) (lanes 2 & 8).

Reaction 2: + protease La (molar ratio of 1:80), - ATP (lanes 3 & 9).

Reaction 3: + protease La (molar ratio of 1:160) & ATP (lanes 4 & 11).

Reaction 4: + protease La (molar ratio of 1:160), - ATP (lanes 5 & 12).

Reactions 5 & 6: + ATP, - protease La (lanes 6, 7, 10, & 13).

After incubation at 37°C for 15h, the mixtures were analysed by SDS-PAGE using a 20% gel, which was stained with Coomassie Blue:

Lanes 1 & 14: BSA, 67,000: β-lactamase, 28,000; β-lactoglobulin, 18,000; cytochrome c, 12,000.

Lanes 2-7: new α-lactalbumin preparation.

Lanes 8-13: original α-lactalbumin preparation.

1 Reaction mixtures with and without ATP (final concentration 3mM) were incubated at 37°C and contained: 40mM Tris/HCl pH 8.0, 20mM $MgCl_2$, about 35μg α-lactalbumin, and protease La (enzyme subunit:α-lactalbumin molar ratio of 1:40), in a final volume of 200μl. At varying times during the incubation, 30μl aliquots were removed from the mixture, SDS added (final concentration 2%), and boiled for 2min. The aliquots were then analysed by SDS-PAGE using a 17.5% gel, which was stained with Coomassie Blue:

Lanes 1 & 16: BSA, 67,000; β-lactamase, 28,000; β-lactoglobulin, 18,000; cytochrome c, 12,000.

Lanes 2, 4, 6, 8, 10 & 12: incubated for 0.5h, 1h, 2h, 3h, 4h, & 8h, respectively, + ATP.

Lanes 3, 5, 7, 9, 11 & 13: incubated as above, - ATP.

Lanes 14 & 15: 5μg α-lactalbumin incubated for 8h (+ATP & -ATP, respectively), - protease La.

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2 Reaction mixtures contained: 30mM Tris/HCl pH 8.0, and about 5μg α-lactalbumin, in a final volume of 30μl. After incubation at 37°C for 6h, the mixtures were analysed by SDS-PAGE using a 15% gel, which was stained with Coomassie Blue:

Lanes 1 & 14: BSA, 67,000; β-lactamase, 28,000; β-lactoglobulin, 18,000; cytochrome c, 12,000.

Lane 2: no additions.

Lane 3: + 25mM $MgCl_2$.

Lane 4: + 3mM ATP.

Lane 5: + 25mM $MgCl_2$ & 3mM ATP.

Lane 6: + protease La (enzyme subunit:α-lactalbumin molar ratio of 1:40).

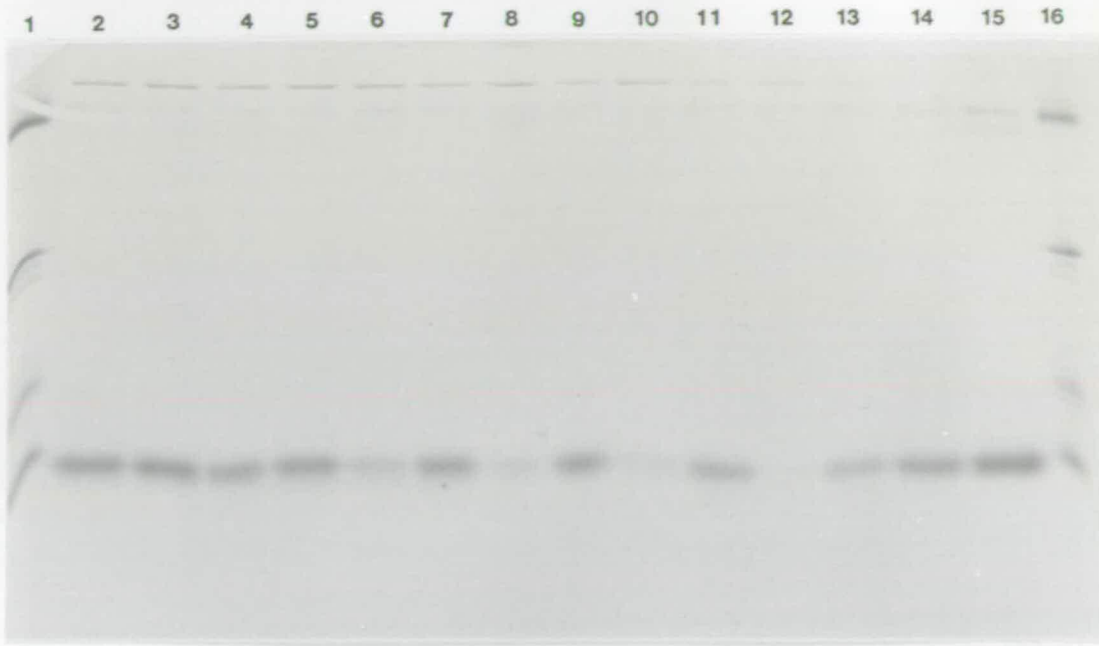
Lane 7: + protease La & 3mM ATP.

Lanes 8, 9, 10, 11, & 12: + protease La, 25mM $MgCl_2$, & 3mM, 1mM, 0.3mM, 0.1mM, or 0.03mM ATP, respectively.

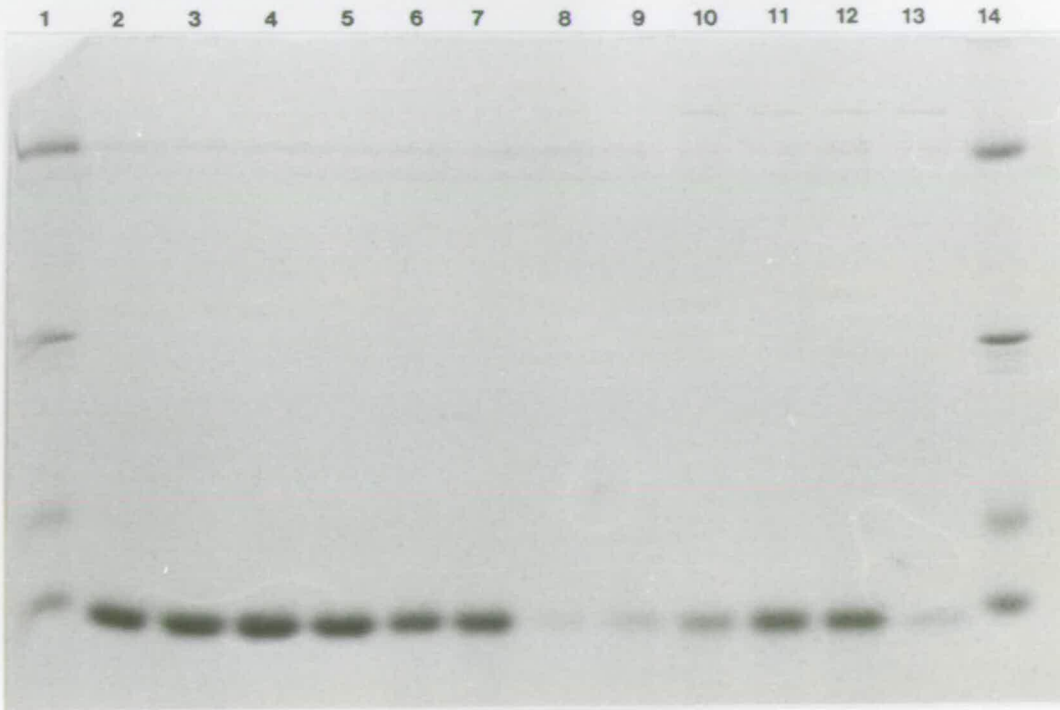
Lane 13: + protease La, 25mM $MgCl_2$, 3mM ATP, & 5μg ml^{-1} ssDNA.

FIGURE 2.28 Studies on the Degradation of α-Lactalbumin By Protease La

1 Time-Course of Degradation



2 Conditions for Degradation



2.3 EFFECT OF DNA ON THE ATP-DEPENDENT PROTEOLYTIC AND ATPase ACTIVITIES OF *E. coli* PROTEASE LA

Protease La possesses multiple activities: it is an ATP-dependent protease (Charette *et al.*, 1981; Chung and Goldberg, 1981), a protein-stimulated ATPase (Charette *et al.*, 1981; Waxman and Goldberg, 1982) and it has a non-specific nucleic acid binding activity (Zehnbauer *et al.*, 1981). The proteolytic and ATPase activities of the protease La preparation were measured in the presence and absence of DNA.

The effect of DNA on the proteolytic activity of protease La was found to be dependent upon the protein substrate (Table 2.11):

1. Single-stranded DNA inhibited the degradation of [^{14}C]-β-lactoglobulin and [^{14}C]-α-lactalbumin.
2. Single- and double-stranded DNA stimulated the digestion of [^{14}C]-β-lactamase.
3. Single- and double-stranded DNA had no effect on the degradation of [^{14}C]-casein.

In a similar experiment, single-stranded DNA was found not to affect the activity of chymotrypsin and trypsin, indicating that the effect of DNA was specific to protease La (Table 2.12). Stimulation of proteolytic activity by single-stranded DNA was detected over a range of [^{3}H]-β-lactamase concentrations: conversely, in assays containing [^{14}C]-α-lactalbumin, inhibition was detected over a range of substrate concentrations (Figure 2.29). Increasing the concentration of either single- or double-stranded DNA led to a greater effect on the proteolytic activity of protease La, whether stimulation (with [^{3}H]- and [^{14}C]-β-lactamase) or inhibition (with [^{14}C]-α-lactalbumin) was examined (Figure 2.29). When [^{14}C]-proteins were digested by protease La in the presence or absence of DNA, large degradation products were not detected by SDS-PAGE (Figure 2.30).

Protease La has an ATPase activity which is stimulated by a protein substrate (Table 2.13). DNA had been found to stimulate and to inhibit the proteolytic activity of protease La, depending upon the protein substrate (Table 2.11). Interestingly, the effect of DNA on ATPase activity, in the presence of protein substrates, was also found to depend upon the protein substrate (Table 2.14). When [^{14}C]-α-lactalbumin was used as a substrate, single-stranded DNA inhibited the protein-stimulated ATPase activity (DNA inhibited the degradation of [^{14}C]-α-lactalbumin by protease La; Table 2.11). In contrast, when [^{14}C]-oxidized-β-lactoglobulin and [^{14}C]-β-lactamase were used as substrates, inhibition by single-stranded DNA of the protein-stimulated ATPase activity was not detected; but there was a suggestion of further stimulation by

single-stranded DNA with [^{14}C]-β-lactamase (DNA stimulated the digestion of [^{14}C]-β-lactamase by protease La, but had no effect on the degradation of [^{14}C]-oxidized-β-lactoglobulin; Table 2.11)

TABLE 2.11 Effect of DNA on the Proteolytic Activity of Protease La

[^{14}C]-PROTEIN	AMOUNT OF DNA ADDED (μg ml^{-1})	PERCENTAGE RADIOLABEL ACID-SOLUBLE [a]	
		+ ssDNA	+ dsDNA
β-lactoglobulin	–	16.0, 14.8	16.0, 14.8
	20	14.3, 10.7	13.9, 16.8
	5	9.8, 12.8	16.7, 17.3
	1	14.2, 13.1	15.3, 16.8
β-lactamase	–	17.2, 17.4	17.2, 17.4
	20	21.9, 20.3	20.8, 18.3
	5	21.5, 20.1	22.8, 24.9
	1	20.1, 17.1	23.8, 29.2
casein	–	24.2, 23.8	24.2, 23.8
	20	23.5, 26.9	25.8, 23.9
	5	23.7, 33.5	23.8, 25.6
	1	25.3, 26.4	27.8, 25.6
α-lactalbumin	–	23.1, 20.1	23.1, 20.1
	20	17.4, 18.4	21.1, 21.7
	5	19.8, 20.5	23.6, 24.2
	1	20.4, 23.5	22.3, 23.9

[^{14}C]-PROTEIN	PERCENTAGE RADIOLABEL ACID-SOLUBLE [a] (– PROTEASE La)
β-lactoglobulin	8.59, 9.11
β-lactamase	14.1, 12.9
casein	14.1, 16.6
α-lactalbumin	11.9, 12.1

a. Assays contained 25mM Tris/HCl pH 8.0, 25mM $MgCl_2$, 3mM ATP, about 2μg [^{14}C]-protein (1–2 x 10^7cpm mg^{-1}), and protease La (enzyme subunit:protein molar ratio of 1:40) in a final volume of 30μl. Double-stranded DNA was pGC1; single-stranded DNA was sonicated calf thymus DNA, boiled for 10min, then placed on ice. After incubation at 37°C for 3h, acid-soluble radioactivity was determined.

TABLE 2.12 Effect of DNA on the Activity of Protease La Compared With Two Other Proteases

[^{14}C]-PROTEIN	ssDNA ($\mu g\ ml^{-1}$)	PERCENTAGE RADIOLABEL ACID-SOLUBLE [a]		
		PROTEASE La	CHYMOTRYPSIN	TRYPSIN
α-lactalbumin	-	42.8, 47.5, 47.8	9.4, 15.4, 16.1	12.8, 12.3, 11.6
	10	25.0, 24.5, 24.1	14.8, 15.3, 14.9	11.9, 12.6, 13.3
	2	37.6, 39.8, 36.7	16.4, 17.2, 16.5	12.3, 12.5, 11.7
oxidized-β-lactoglobulin	-	28.7, 29.0, 28.6	49.1, 52.6, 46.4	34.3, 34.6, 34.2
	10	26.6, 26.3, 26.6	56.7, 54.5, 57.8	37.4, 36.7, 35.7
	2	28.4, 28.7, 25.8	54.0, 52.2, 52.8	31.3, 34.5, 36.2
β-lactamase	-	11.4, 11.2, 10.3	31.7, 35.6, 35.4	19.0, 19.2, 19.3
	10	13.6, 14.9, 16.2	30.5, 28.9, 31.3	19.7, 20.2, 22.0
	2	15.6, 15.5, 17.1	32.7, 29.9, 31.0	19.2, 18.6, 19.8

[^{14}C]-PROTEIN	PERCENTAGE RADIOLABEL ACID-SOLUBLE [a] (- PROTEASE La)
α-lactalbumin	11.7, 12.5, 12.7
oxidized-β-lactoglobulin	13.2, 14.3, 15.5
β-lactamase	10.8, 10.3, 11.1

a. Assays contained 25mM Tris/HCl pH 8.0, 25mM $MgCl_2$, 3mM ATP, and about 2μg [^{14}C]-protein ($1-2 \times 10^7$cpm mg^{-1}) in a final volume of 30μl. Protease La, chymotrypsin, and trypsin were added at a protease subunit:protein molar ratio of 1:40. Single-stranded DNA was sonicated calf thymus DNA, boiled for 10min, then placed on ice. After incubation at 37°C for 3h, acid-soluble radioactivity was determined.

FIGURE 2.29 Effect of DNA on the Proteolytic Activity of Protease La

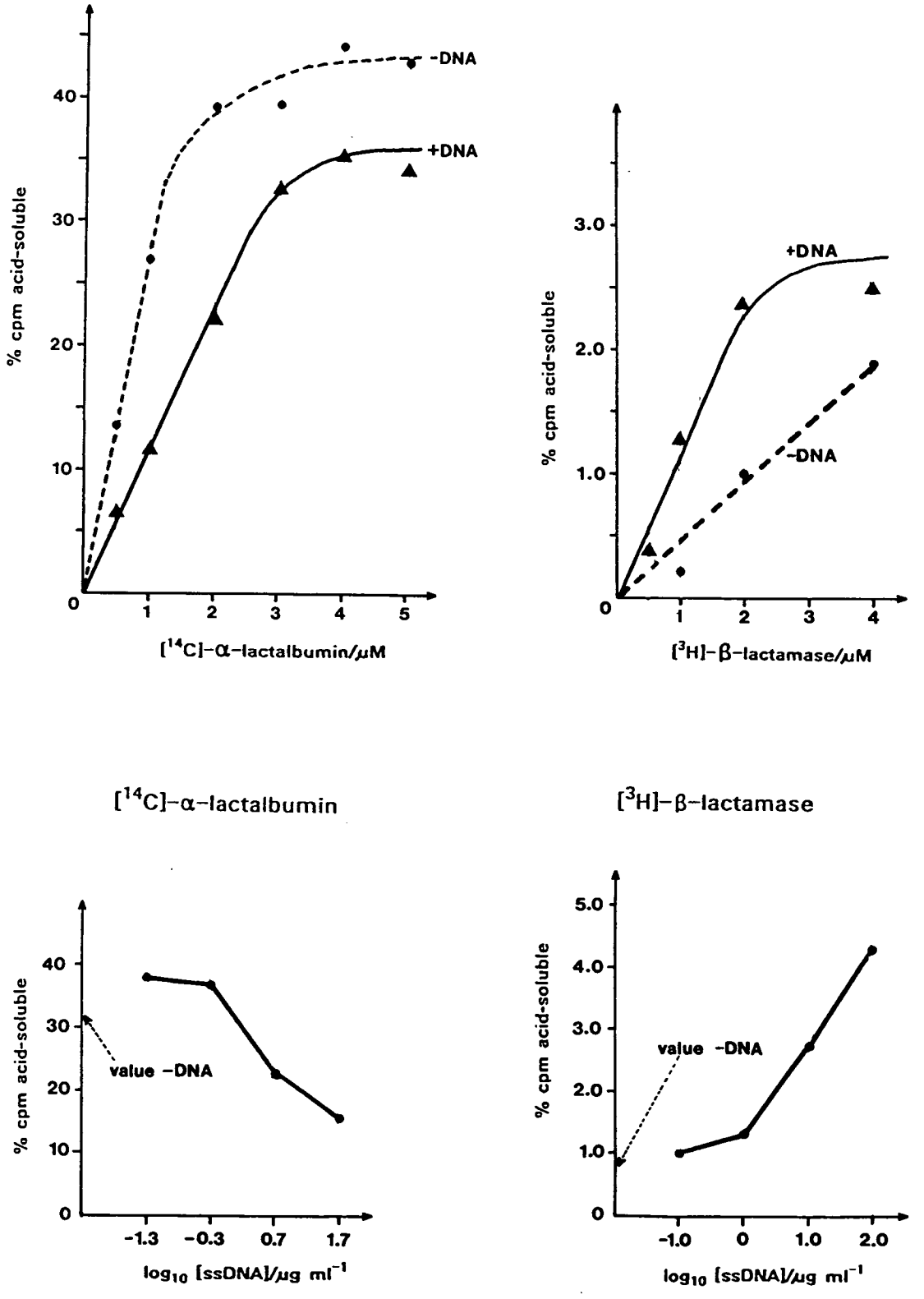
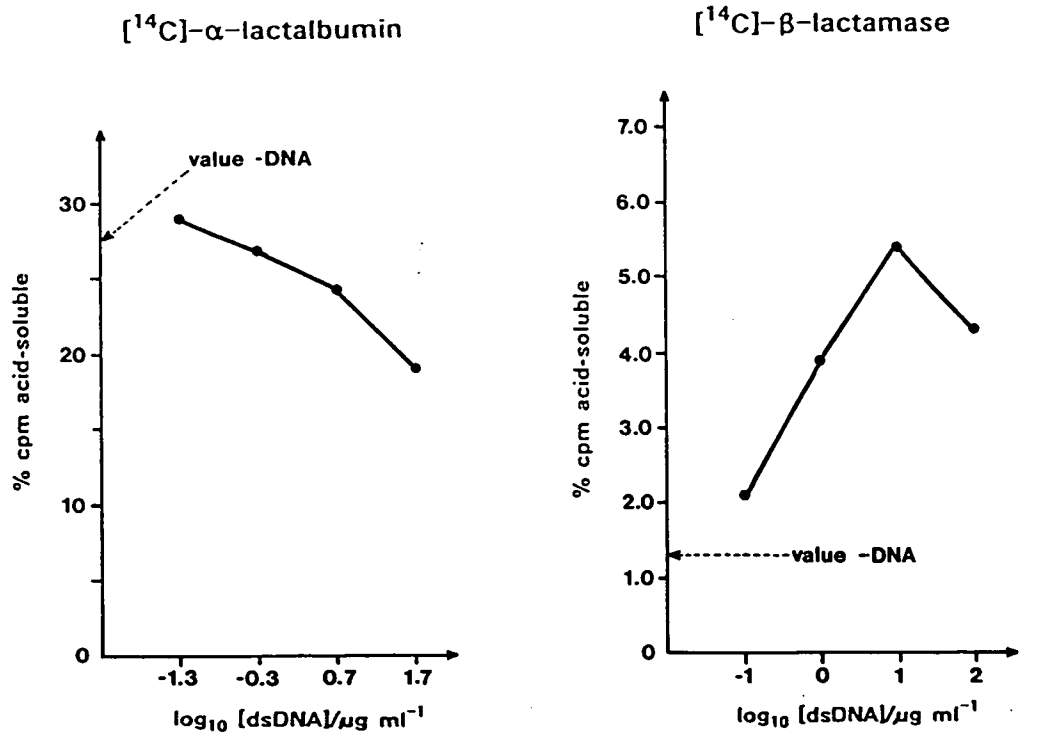


FIGURE 2.29 (continued)



Each assay contained 25mM Tris/HCl pH 8.0, 25mM $MgCl_2$, 3mM ATP, and protease La (enzyme subunit:protein molar ratio of 1:40, except in the case of A & B), in a final volume of 30μl. The different reactions were as follows:

Reactions A & B: varying concentrations of [^{14}C]-α-lactalbumin (1.5 x 10^7cpm mg^{-1}) and [^{3}H]-β-lactamase (2.4 x 10^7cpm mg^{-1}) were incubated with 0.7μg protease La, in the presence or absence of ssDNA (final concentration 5μg ml^{-1}).

Reactions C & D: varying concentrations of ssDNA were incubated with about 2μg of either [^{14}C]-α-lactalbumin (1.5 x 10^7cpm mg^{-1}) or [^{3}H]-β-lactamase (2.4 x 10^7cpm mg^{-1}).

Reactions E & F: varying concentrations of dsDNA were incubated with about 2μg of either [^{14}C]-α-lactalbumin (1.0 x 10^7cpm mg^{-1}) or [^{14}C]-β-lactamase (0.8 x 10^6cpm mg^{-1}).

After incubation at 37°C for 3-4h, acid-soluble radioactivity was measured. Values shown are the mean of triplicate assays, corrected for acid-soluble radioactivity in the control (no enzyme). ssDNA was sonicated calf thymus DNA, boiled for 10min, then placed on ice; dsDNA was pGC1.

Reaction mixtures contained: 25mM Tris/HCl pH 8.0, 25mM $MgCl_2$, 3mM ATP, about 5μg of either [^{14}C]-α-lactalbumin (1.5 x 10^7cpm mg^{-1}) (A), or [^{14}C]-β-lactamase (1.0 x 10^7cpm mg^{-1}) (B), and protease La (enzyme subunit:protein molar ratios of either 1:40 (A), or 1:13 (B)), in a final volume of 30μl. In addition, some reaction mixtures contained varying amounts of either ssDNA (sonicated calf thymus DNA, boiled for 10min, then placed on ice), or dsDNA (pGC1 DNA). After incubation at 37°C for 3h, the mixtures were analysed by SDS-PAGE using either 15% (A), or 12.5% (B) gels, which were stained with Coomassie Blue. For both A and B, the gels contained:

Lanes 1 & 14: BSA, 67,000; β-lactamase, 28,000; β-lactoglobulin, 18,000; cytochrome c, 12,000.

Lane 2: + 10μg ml^{-1} ssDNA, - protease La.

Lanes 3 & 12: - ATP.

Lanes 4 & 11: no additions.

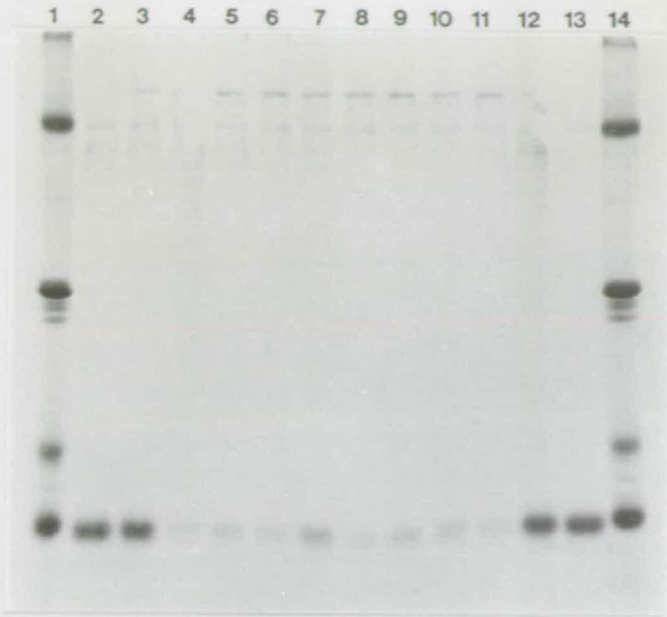
Lanes 5-7: + ssDNA (0.1, 1.0, & 10μg ml^{-1}, respectively).

Lanes 8-10: + dsDNA (0.1, 1.0, & 10μg ml^{-1}, respectively).

Lane 13: + 10μg ml^{-1} dsDNA, - protease La.

FIGURE 2.30 Effect of DNA on the Degradation of [^{14}C]-Proteins By Protease La

A [^{14}C]-α-Lactalbumin



B [^{14}C]-β-Lactamase

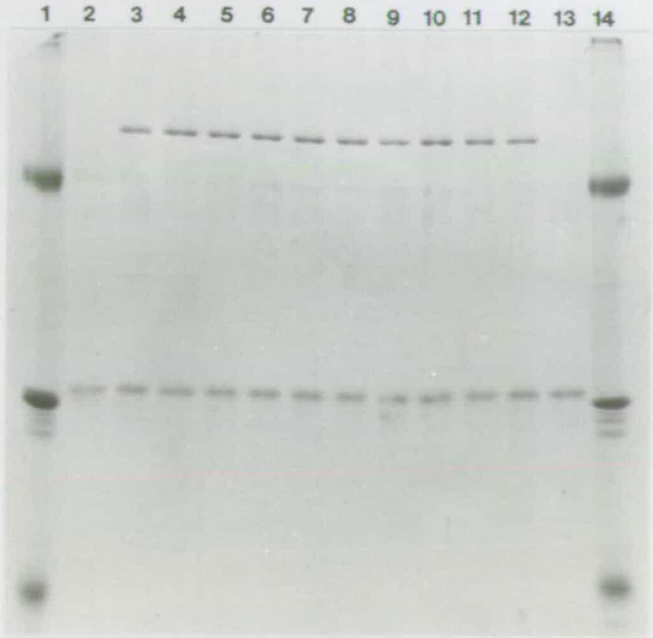


TABLE 2.13 Effect of DNA on the ATPase Activity of Protease La

ssDNA	α-LACTALBUMIN	[γ-^{32}P] ATP HYDROLYSED (nmol) [a]	
		+ PROTEASE La	- PROTEASE La
-	-	2.93, 2.90	0.794, 0.731
+	-	2.30, 2.52	0.747, 0.720
-	+	5.43, 5.37	0.794, 0.750
+	+	5.25, 5.15	0.799, 0.812

a. Reaction mixtures contained 50mM Tris/HCl pH 8.0, 25mM $MgCl_2$, 0.1mM [γ-^{32}P] ATP (about 28cpm $pmol^{-1}$), and 2.5μg protease La in a final volume of 50μl. Single-stranded DNA (sonicated calf thymus DNA, boiled for 10min, then placed on ice) at a final concentration of 10μg ml^{-1}, and 16μg α-lactalbumin were added, as indicated. After incubation at 37°C for 1h, ATP hydrolysis was measured (Goldmark & Linn, 1972).

TABLE 2.14 Effect of DNA on the ATPase Activity of Protease La in the Presence of Protein Substrates

[^{14}C]-PROTEIN	ssDNA	[γ-^{32}P] ATP HYDROLYSED (nmol) [a]	
		+ PROTEASE La	- PROTEASE La
EXPERIMENT 1 [a]			
-	-	9.20, 9.28, 8.34	1.89, 1.85, 1.84
	+	8.53, 8.50, 8.26	1.91, 1.98, 1.83
α-lactalbumin	-	10.7, 11.6, 11.4	-
	+	10.1, 10.2, 10.2	-
β-lactamase	-	11.6, 10.7, 10.4	-
	+	11.1, 12.2, 11.4	-
oxidized-β-lactoglobulin	-	12.0, 12.0, 12.3	-
	+	11.3, 11.7, 12.4	-
EXPERIMENT 2 [b]			
-	-	7.46, 7.81, 7.16	3.33, 3.07
	+	7.46, 7.10, 6.28	2.79, 2.99
α-lactalbumin	-	14.9, 13.5, 15.2	3.69, 3.55
	+	10.4, 11.2, 11.2	3.74, 3.78
β-lactamase	-	9.57, 8.37, 7.77	3.22, 2.91
	+	8.56, 8.40, 8.99	3.15, 3.02

a. Reaction mixtures contained 25mM Tris/HCl pH 8.0, 25mM $MgCl_2$, 0.5mM [γ-^{32}P] ATP (about 25cpm pmol^{-1}), and 0.35μg protease La in a final volume of 30μl. Single-stranded DNA (sonicated calf thymus DNA, boiled for 10min then placed on ice) was added to a final concentration of 2μg ml^{-1}. Protein substrates (1-2 x 10^7 cpm mg^{-1}) were added at a 40-fold molar excess relative to the amount of protease La subunit. After incubation at 37°C for 3h, ATP hydrolysis was measured (Goldmark & Linn, 1972).

b. Reaction mixtures were as above, except that 0.5mM [γ-^{32}P] ATP (about 15cpm pmol^{-1}) and a final concentration of 5μg ml^{-1} ssDNA were used, and the assays incubated for 2h.

CHAPTER 3

Discussion

3.1 THE SPECIFICITY OF THE *E. coli* ATP-DEPENDENT PROTEASE La

Protease La was purified from an over-expressing strain of *E. coli* using published procedures (Figures 2.5, 2.6, 2.7). A band of molecular weight 90,000, corresponding in size to one subunit of protease La (87,000; Chin *et al.*, 1988), was the major species detected by SDS-PAGE (Figure 2.9). The estimated molecular weight and the ATP-dependent proteolytic activity demonstrated by this preparation (Table 2.3) suggest that it is indeed protease La. Strong evidence in support of this came from N-terminal sequencing, which confirmed that the first ten residues of the preparation were identical to the predicted amino acid sequence of the *lon* gene product (Chin *et al.*, 1988). The preparation was assessed to be free from contamination by non-ATP-dependent proteases since in many assays proteolytic activity in the absence of ATP was not detected (Table 2.3). The very low level of activity observed in the absence of ATP in a few assays was thought to be insignificant because in subsequent assays containing much greater amounts of the preparation, this activity was not detected (Table 2.3). However, contamination by ATP-dependent proteases cannot be discounted. The specific activity of protease La was 1.3mg casein hydrolysed h^{-1} mg^{-1} enzyme (Table 2.3). This value compares favourably with the specific activity (0.7mg casein hydrolysed h^{-1} mg^{-1} enzyme) of the preparation of Chung and Goldberg (1981), but is less than that of other preparations (specific activities ranging from 2.0-2.6: Waxman and Goldberg, 1982; Chung and Goldberg, 1982; Charette *et al.*, 1984). The turnover number for [^{14}C]-casein could not be determined because the number of peptide bonds cleaved by protease La was not known. However, other assays have been used to calculate turnover numbers for several substrates (Maurizi, 1987; Menon *et al.*, 1987). It will be interesting to compare turnover numbers of protease La with those of other intracellular proteases.

Optimum conditions for protease La were determined by measuring the amount of [^{14}C]-β-lactoglobulin rendered acid-soluble under varying conditions and were found to be (Figure 2.10):

1. pH 9.0-9.5.
2. Low ionic strength.
3. Substrate concentration greater than 4μM (twice apparent K_m).
4. 1-3mM ATP.
5. 5-25mM Mg^{2+} ion.
6. In the absence of DNA.

These conditions for maximal activity are in good agreement with those determined in assays using [^{3}H]-casein (Larimore *et al.*, 1982; Waxman and Goldberg, 1985). Interestingly, at a high concentration of ATP (9mM) the activity of protease La was lower than at the optimum concentration. The reason for this effect is not understood, but it may have been caused by depletion of the Mg^{2+} ion pool by ATP. Similarly, at a high concentration of Mg^{2+} ion (50mM) activity was lower than at the optimum concentration. The reason for this is not understood either, but it may have been a reflection of the low ionic strength requirement of protease La. The effect of DNA will be discussed later.

During an investigation into the degree of specificity shown by protease La, proteins were incubated with the protease using the optimum conditions described above, except a pH (7.7 at 37°C) closer to physiological pH was used to ensure that substrates retained a normal conformation. Initially degradation of radiolabelled proteins was monitored but this work was extended to include native proteins.

Studies of intracellular proteolysis in *E. coli* suggest that protease La is highly specific. Since highly specific endopeptidases isolated from *E. coli* (RecA protein and signal peptidases I and II) hydrolyse only a single peptide bond of their substrates (Pabo *et al.*, 1979; Horri *et al.*, 1981; Zwizinski and Wickner, 1980; Tokunago *et al.*, 1982) it seemed likely that protease La would cleave only one, or a limited number of peptide bonds of radiolabelled proteins. The products of radiolabelled proteins digested by protease La were examined by SDS-PAGE and HVPE. Surprisingly, large degradation products were not detected by SDS-PAGE, despite using a staining procedure designed to prevent "washout", but many radiolabelled peptides were detected by HVPE (Figure 2.11). This finding indicates that several peptide bonds of each substrate had been hydrolysed by protease La and is in agreement with the results of Menon *et al.* (1987) and Maurizi (1987).

The digestion of [^{14}C]-α-lactalbumin in the absence of ATP (Figures 2.11, 2.12) was unexpected because protease La has been reported to be dependent upon ATP (Chung and Goldberg, 1981; Charette *et al.*, 1981). The results of many assays of protease La reported in this thesis are in accord with this finding (Table 2.3). However, protease La has also been reported to possess an ATP-independent proteolytic activity (Waxman and Goldberg, 1985; 1986; Goldberg and Waxman, 1985). This activity may be an artefact induced by purification or storage at -70°C (Waxman and Goldberg, 1985). Protease La purified in the present investigation may also have lost its dependence upon ATP (to become an ATP-stimulated protease) on storage at

-70°C. This seems the most likely explanation for the degradation of [^{14}C]-α-lactalbumin by protease La in the absence of ATP, and other possible explanations given before are probably incorrect (Section 2.2.2).

An intermediate in the degradative pathway of [^{14}C]-α-lactalbumin by protease La may have been identified. A polypeptide of molecular weight about 11,000 was detected in a reaction mixture containing [^{14}C]-α-lactalbumin, protease La and ATP, and in a similar mixture which lacked ATP (Figures 2.11, 2.12). A greater amount of the polypeptide and of [^{14}C]-α-lactalbumin were detected in the mixture lacking ATP than in the mixture containing ATP. Assuming that the preparation of protease La was not contaminated by proteases (discussed above), and that the preparation of [^{14}C]-α-lactalbumin was homogeneous, this finding indicates that the polypeptide was not an end-product of the degradative pathway. More work is needed to characterize this polypeptide and other products to allow elucidation of the degradative pathway of [^{14}C]-α-lactalbumin by protease La.

Although non-hydrolysable analogues of ATP were found to be unable to support digestion of radiolabelled proteins by protease La (Charette *et al.*, 1981; Chung and Goldberg, 1981), they can support peptide bond cleavage (Goldberg and Waxman, 1985). To explain this paradox, Goldberg and Waxman (1985) proposed a cyclical mechanism for the degradation of a protein by protease La: binding of ATP allosterically activates the enzyme to a form capable of hydrolysing peptide bonds; peptide bond cleavage occurs generating large products; ATP is hydrolysed, which allows the release of products, returning the protease to the inactive form. To digest a protein to acid-soluble fragments many rounds of the cycle are required, although in a single round one peptide bond can be cleaved without ATP hydrolysis. Degradation of a protein by protease La in the presence of a non-hydrolysable analogue of ATP is blocked at the last step of the cycle, preventing the release of products. In support of this model, Goldberg (pers. comm.) reported that incubation of [^{3}H]-casein, protease La and non-hydrolysable analogues of ATP led to the generation of high molecular weight degradation products, presumably the result of cleavage of a single peptide bond. However, in opposition to the model, Maurizi (1987) reported that in addition to ATP, non-hydrolysable analogues could support digestion of λ*N* product by protease La giving identical products to those obtained with ATP.

In view of the controversy, the degradation of radiolabelled proteins by protease La in the presence of non-hydrolysable analogues of ATP (AMP-PCP and AMP-PNP) was investigated during this project. Compared to the digestion of three

radiolabelled protein preparations in the presence of ATP, the degradation detected with AMP-PCP was insignificant, and only a very small amount of one of the preparations was digested with AMP-PNP (Table 2.6). In this experiment a molar ratio of enzyme subunit to substrate of 1:40 was used. Consequently, if the cyclical mechanism proposed by Goldberg and Waxman (1985) is correct, then only 1/40th of radiolabel could possibly have been rendered acid-soluble (assuming that a single peptide bond is cleaved per subunit of protease La, and that degradation products are acid-soluble). Such a low level of radiolabel could not be reliably detected over background levels so a much greater molar ratio of 1:1 was used in a subsequent experiment. Despite this, the digestion of [^{14}C]-α-lactalbumin detected in the presence of AMP-PCP was insignificant, and only minimal degradation with AMP-PNP was observed, compared to the level with ATP (Table 2.7). Because of the strong possibility that digestion of proteins by protease La in the presence of non-hydrolysable analogues of ATP would generate acid-insoluble fragments, degradation was also monitored by SDS-PAGE. Digestion of [^{14}C]-α-lactalbumin was detected only in the presence of ATP and not with AMP-PCP and AMP-PNP no matter whether a molar ratio of 1:40 or 1:1 was used (Figure 2.13). Thus under these experimental conditions non-hydrolysable analogues did not support digestion of radiolabelled proteins by protease La, although ATP did. Clearly, more work needs to be carried out to determine why the peptide bonds of λ*N* product and small peptides, but not those of radiolabelled proteins, are cleaved by protease La in the presence of non-hydrolysable analogues of ATP (Maurizi, 1987; Goldberg and Waxman, 1985; Chung and Goldberg, 1981; Charette *et al.*, 1981; this thesis). However, if it does become accepted that non-hydrolysable analogues of ATP can support protein degradation by protease La, then the cyclical mechanism proposed by Goldberg and Waxman (1985), and extended by Menon and Goldberg (1987b), will have to be amended.

Protease La may possess a highly selective proteolytic activity *in vivo* (Section 1.6). When nineteen native protein preparations were incubated with protease La *in vitro*, only two (bovine α-lactalbumin and casein) were completely digested and two others (chick erythrocyte histone and horse heart cytochrome *c*) were partially degraded (Figures 2.19, 2.23, 2.24, 2.25). Therefore, protease La has a highly selective proteolytic activity *in vitro*. This finding is in agreement with the results of Maurizi (1987). Large degradation products were not detected suggesting that several peptide bonds were hydrolysed in each substrate. This discovery is in accordance with the results obtained when the degradation of radiolabelled proteins was investigated and with published results (Menon *et al.*, 1987; Maurizi, 1987). Interestingly, single-stranded DNA inhibited the digestion of the preparations of α-lactalbumin and histone

(Figure 2.23). The effect of DNA will be discussed later.

Conditions for the degradation of the preparation of α-lactalbumin by protease La were investigated:

1. The preparation was cleaved by catalytic amounts of protease La (Figure 2.26.1).

2. Digestion of the preparation increased with length of incubation (Figure 2.28.1).

Digestion of α-lactalbumin by protease La in the absence of ATP was observed (Figure 2.26). As discussed earlier, the most likely explanation for this finding is that the proteolytic activity of protease La purified during this project was stimulated by and not dependent upon ATP, and that this behaviour was only manifest after storage at -70°C for several weeks. A degradation product of molecular weight about 10,000 was detected in one preparation of α-lactalbumin (Figure 2.26) but not in another (Figure 2.27). The reason for this discrepancy is not understood. A greater amount of the polypeptide was detected when α-lactalbumin was incubated with protease La in the absence of ATP than in its presence (Figures 2.26, 2.27). Since a precursor/product relationship does not hold for this polypeptide it is probably an intermediate in the degradative pathway of some preparations of α-lactalbumin by protease La. A similar product was observed when [^{14}C]-α-lactalbumin was digested by protease La, suggesting that native and [^{14}C]-α-lactalbumin share a common degradative pathway.

A preliminary investigation into the features of proteins that confer sensitivity to protease La revealed that heat denaturation of five protein preparations was insufficient to convert them into substrates although more drastic denaturation of a β-lactoglobulin preparation did (Figures 2.24, 2.25). However, the extent of degradation of drastically denatured β-lactoglobulin was much less than that of preparations of native α-lactalbumin and casein (Figures 2.23, 2.25). These results are in accord with the findings of Maurizi (1987). Waxman and Goldberg (1986) reported that for some proteins, but not others, extensive denaturation rendered them susceptible to digestion by protease La. Clearly, denaturation does not necessarily confer on a protein features that are recognized by the protease. Approaches which have been followed to elucidate the nature of these features are discussed below.

The selectivity shown by protease La may arise from its ability to recognize specific structural features or sequences present on the surface of only a few proteins. For some proteins these features can be exposed by denaturation. Using a series of fluorogenic peptides Waxman and Goldberg (1985) reported that protease La preferentially cleaves naphthylamide bonds of tetrapeptides in which the naphthyl

group is attached to the carboxyl group of phenylalanine. Fluorogenic peptides containing this bond were also substrates of chymotrypsin. However, substrates of chymotrypsin were not all hydrolysed by protease La indicating that protease La has a more restricted specificity than chymotrypsin. Further study revealed that protease La preferentially cleaves peptides which:

1. Contain at least four peptide bonds.
2. Are hydrophobic in character.
3. Have a negatively charged blocking group at the amino terminus.

This study suggests that substrates of protease La possess primary sequences that are recognized by the active site of the protease. In another study the peptide bond specificity of protease La was investigated using two polypeptides, glucagon and oxidized insulin B chain, and a physiological substrate, λ*N* product (Maurizi, 1987). The peptide bonds hydrolysed in these substrates are shown in Figure 3.1. In the majority of the bonds cleaved the carboxyl-donating amino acid has an aliphatic side chain (usually Leu or Ala). However, at three sites of cleavage the carboxyl-donating amino acid has a non-aliphatic side chain (Ser or Cys). Many peptide bonds within these substrates that are not hydrolysed have carboxyl-donating amino acids with these side chains. Comparison of the primary sequence flanking the susceptible bonds reveals no obvious similarity which could explain the selectivity shown by protease La. Therefore, additional features of these substrates must play important roles in determining susceptibility to degradation by protease La.

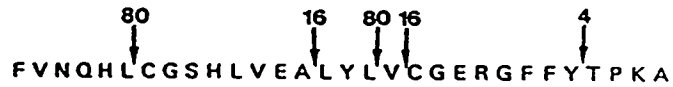
Structural features of proteins may be important in determining sensitivity to protease La. No work has been reported concerning this topic. An approach which could be followed is to determine which peptide bonds are hydrolysed in several proteins of known tertiary structure: comparison of the sites of cleavage may reveal a structural motif in common. At present this approach is limited by the number of suitable proteins: the tertiary structures of substrates of protease La (λ*N* product, bovine α-lactalbumin) have not been elucidated, and proteins of known structure may not be substrates (such as haemoglobin or cytochromes).

Approaches which have been used to determine the features of proteins recognized by protease La have assumed that specificity resides in the active (catalytic) site of the protease. However, studies with model fluorogenic peptides suggest that substrates bind to protease La at two sites (regulatory and catalytic; Waxman and Goldberg, 1986). Currently it is uncertain whether the specificity shown by protease La resides in one, or other, or both of these sites. If proteolysis does

occur by a cyclical mechanism (Goldberg and Waxman, 1985; Menon and Goldberg, 1987b) then the regulatory site must have a role in conferring specificity since substrates bind to this site leading to activation of the catalytic site. In addition, the finding that several peptide bonds of substrates are hydrolysed by protease La may be a reflection that specificity resides primarily in the regulatory site. Hence future work must embrace the possibility that both sites confer specificity to protease La.

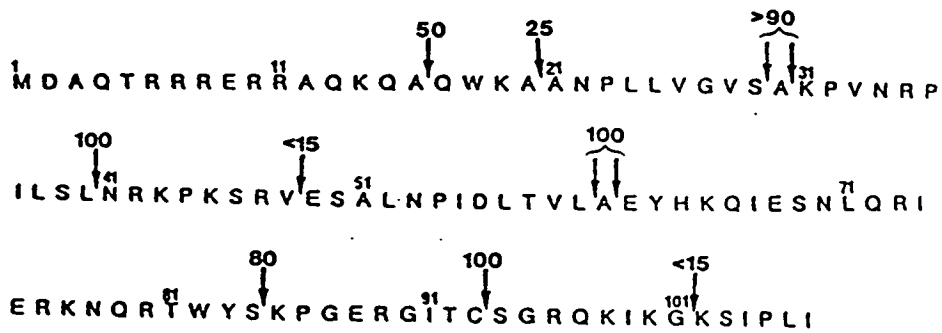
FIGURE 3.1 Peptide Bonds Cleaved by Protease La

A Sites of Cleavage in Oxidized Insulin B Chain



B Sites of Cleavage in Glucagon

C Sites of Cleavage in λ *N* Product



Arrows indicate the bonds hydrolysed. **Numbers** above the arrows indicate the percentage of the original molecules digested at that bond.

Taken from Maurizi (1987).

3.2 THE EFFECT OF DNA ON THE PROTEOLYTIC AND ATPase ACTIVITIES OF THE *E. coli* ATP-DEPENDENT PROTEASE La

Protease La possesses multiple activities *in vitro*: it is an ATP-dependent protease (Charette *et al.*, 1981; Chung and Goldberg, 1981), a protein-stimulated ATPase (Charette *et al.*, 1981; Waxman and Goldberg, 1982), and it has a non-specific nucleic acid binding activity (Zehnbauer *et al.*, 1981). Three independent studies of protease La (Charette *et al.*, 1984; Chung and Goldberg, 1982; this thesis) have reported the effect of DNA on these activities and conflicting results have been obtained. The areas of conflict and possible explanations are described below.

A central feature of the results of Charette *et al.* (1984) is their finding that DNA stimulates the ATPase activity of protease La in the absence of protein substrates. Based on this observation they proposed that protease La has two independent enzymic activities: a DNA-stimulated ATPase activity, and a proteolytic activity which is obligatorily coupled to a protein-stimulated ATPase. However, stimulation of ATPase activity by DNA was not reported by Chung and Goldberg (1982). They suggested that the preparation of protease La used by Charette *et al.* (1984) was contaminated by one of several DNA-associated ATPases found in *E. coli*. However, contamination by a DNA-associated ATPase does not explain the inhibition of the DNA-stimulated ATPase activity by CapR9 protein reported by Charette *et al.* (1984). The *lon R9* allele in *E. coli* codes for an altered form of protease La, the CapR9 protein, which has lost ATPase and proteolytic activities but retained non-specific nucleic acid binding activity (Charette *et al.*, 1981; Zehnbauer *et al.*, 1981). Mixing of mutant and wild-type proteins *in vitro* suggested that the CapR9 protein intercalates into the multimeric form of the wild-type protein and specifically inhibits ATPase and proteolytic activities in a pattern consistent with disruption of the multimer (Charette *et al.*, 1982; Chung *et al.*, 1983). Consequently, inhibition by CapR9 protein is strong evidence that protease La has a DNA-stimulated ATPase activity.

The effect of DNA on the ATP-dependent proteolytic activity of protease La is also controversial. Charette *et al.* (1984) reported that at a low substrate concentration (0.1μM [^{3}H]-casein) single- and double-stranded DNA inhibited proteolytic activity. However, the inhibition by DNA could be relieved by using a higher substrate concentration (2μM). This finding led them to suggest that there is competition between DNA and protein for binding to protease La giving support to their proposal that protease La has two independent enzymic activities (DNA-stimulated ATPase and an ATP hydrolysis-dependent proteolytic activity). In contrast to these results, Chung and Goldberg (1982) reported that single- and

double-stranded DNA stimulated proteolytic activity at substrate concentrations greater than 0.3μM [^{3}H]-casein. They also reported that single- and double-stranded DNA inhibited activity at low substrate concentrations (less than 0.3μM). Depending upon the radiolabelled protein used in assays, single-stranded DNA is reported in this thesis to stimulate ([^{14}C]-β-lactamase), inhibit ([^{14}C]-α-lactalbumin and [^{14}C]-β-lactoglobulin) or have no effect on ([^{14}C]-casein) the proteolytic activity of protease La (Table 2.11). Surprisingly, in the same experiment inhibition by double-stranded DNA was not detected although it did stimulate proteolytic activity depending upon the substrate (Table 2.11). The reason for this finding is not understood. Further studies using [^{14}C]-α-lactalbumin revealed that single-stranded DNA inhibited proteolytic activity over a range of substrate concentrations (0.5-5μM) with greatest inhibition occurring at 1-2μM (Figure 2.29). Conversely, studies using [^{3}H]-β-lactamase revealed that single-stranded DNA stimulated activity over a range of substrate concentrations (0.5-4μM) with the greatest stimulation occurring at 1-2μM (Figure 2.29). It must be noted, however, that a DNA-induced modification of substrates, which alters their susceptibility to digestion by protease La, cannot be discounted by the results presented in this thesis (or by Chung and Goldberg, 1982).

It is difficult to rationalize the conflicting reports concerning the effect of DNA on the proteolytic activity of protease La. Charette *et al.* (1984) suggested that the stimulation reported by Chung and Goldberg (1982) was in fact stabilization. However, stimulation by DNA is also reported in this thesis, despite adding protease La to assays last, so this explanation seems unlikely. A possible explanation for the failure of Charette *et al.* (1984) to detect stimulation by DNA follows from the results described in this thesis, which indicate that the effect of DNA depends upon the substrate used in assays. There is general agreement that DNA inhibits proteolytic activity at low substrate concentrations. Charette *et al.* (1984) suggested that inhibition by DNA at low substrate concentrations (0.1μM [^{3}H]-casein) was caused by the competition between the substrates for binding to protease La. However, inhibition by DNA is also reported in this thesis at very much higher substrate concentrations (0.5-5μM [^{14}C]-α-lactalbumin). Moreover, although increasing the concentration of single- and double-stranded DNA did lead to greater inhibition of the degradation of [^{14}C]-α-lactalbumin, it also led to greater stimulation of the digestion of [^{14}C]- and [^{3}H]-β-lactamase by protease La (Figure 2.29). These results and another report of stimulation of activity by DNA (Chung and Goldberg, 1982) suggest that competition between substrates for binding to protease La is not the reason for inhibition by DNA. However, evidence to support this proposal is given by the finding that the rate of degradation of [^{3}H]-casein by protease La depends upon the

concentration of DNA in a complex fashion: from 0-2μg ml^{-1} proteolytic activity increased rapidly; from 40-200μg ml^{-1} activity remained constant; and from 40-200μg ml^{-1} activity decreased slowly to a level lower than without DNA (Chung and Goldberg, 1982). The reason for this complex behaviour is not understood but the inhibition observed at high concentrations of DNA does suggest competition between DNA and [^{3}H]-casein for binding to protease La. An alternative explanation for inhibition by DNA at low substrate concentrations was given by Chung and Goldberg (1982). They suggested that this effect was caused by a cooperative interaction between the subunits of protease La in the presence of DNA. This proposal explains their observation that DNA inhibits proteolytic activity at low substrate concentrations (<0.3μM [^{3}H]-casein) but stimulates activity at higher substrate concentrations (>0.3μM). However, inhibition by DNA is not reported in this thesis in assays using [^{3}H]-β-lactamase although stimulation was observed with this substrate (Figure 2.29). Possibly the substrate concentrations used (0.5-4μM) were too high to detect inhibition by DNA. The data of proteolytic activity at different concentrations of [^{3}H]-β-lactamase presented in this thesis does not allow conclusions concerning the nature of the dependency of proteolytic activity on substrate concentration in the presence of DNA to be reached. However, for [^{14}C]-α-lactalbumin the dependency of activity on substrate concentration is hyperbolic in the presence of DNA (Figure 2.29). Thus, the results reported in this thesis do not support the proposal that inhibition by DNA is caused by a cooperative interaction between the subunits of protease La in the presence of DNA.

DNA is reported in this thesis to stimulate, inhibit or have no effect on the proteolytic activity of protease La measured at high substrate concentrations (Figure 2.29, Table 2.11). A possible explanation for this finding is that binding of DNA alters the specificity of protease La. Evidence to support this proposal is lacking. When [^{14}C]-α-lactalbumin and [^{14}C]-β-lactamase were incubated with protease La in the presence and absence of DNA, degradation products were not detected by SDS-PAGE (Figure 2.30). Similarly, when digestion of native proteins was monitored no products were observed (Figure 2.23). Unfortunately, Maurizi (1987) did not report the effect of DNA on the digestion of λ*N* product by protease La. An alternative explanation for the effect of DNA reported in this thesis is that binding of DNA alters the rate of hydrolysis of the same peptide bonds within a substrate. Evidence to support this proposal is given by the finding that DNA stimulates the hydrolysis of a naphthylamide bond of a fluorogenic peptide (Waxman and Goldberg, 1985). However, there are no reports of DNA decreasing the rate of hydrolysis of a peptide (or naphthylamide) bond.

At present, it is not understood how DNA exerts its effect on protease La. In due course, characterization of the polypeptides generated when proteins are digested by protease La in the presence and absence of DNA will clarify the situation. To determine the physiological significance of protease La binding to DNA this problem must be solved. At present two roles can be envisaged. Firstly, protease La may possess two different highly specific proteolytic activities *in vivo*: one when bound to the chromosome, the other when free in the cytosol. Alternatively, the location of a substrate may determine its rate of degradation by protease La: some proteins may be rapidly digested only when they are bound to the chromosome, others may be rapidly degraded only when they are in the cytosol.

There is conflict over the effect of DNA on the ATPase activity of protease La when this activity is measured in the presence of protein substrates. Chung and Goldberg (1982) reported that in the presence of [^{3}H]-casein (25μM) ATPase activity was stimulated by DNA. Since DNA also stimulated the ATP-dependent degradation of this substrate they proposed that protease La has a single enzymic activity: a proteolytic activity, obligatorily coupled to a protein-stimulated ATPase, which is stimulated by DNA. Charette *et al.* (1984) also reported that DNA stimulated the ATPase activity of protease La in the presence of [^{3}H]-casein (0.2μM). However, since DNA inhibited the ATP-dependent degradation of this substrate, this result is support for their proposal that protease La has two independent enzymic activities (a DNA-stimulated ATPase and a proteolytic activity obligatorily coupled to a protein-stimulated ATPase) which function simultaneously. In contrast to this finding reported by Charette *et al.* (1984), DNA is reported in this thesis to inhibit ATPase activity when the activity is measured in the presence of a substrate ([^{14}C]-α-lactalbumin, 5μM) whose ATP-dependent degradation by protease La was inhibited by DNA (Table 2.14). This result is in accordance with the failure to detect stimulation by DNA of ATPase activity (in the absence of a protein substrate), and it is consistent with the proposal of Chung and Goldberg (1982) that protease La has a single enzymic activity, although it now appears that DNA has a complex effect on this activity. Supporting evidence for this proposal is given by:

1. The discovery that DNA had no effect on ATPase activity when this activity was measured in the presence of a substrate ([^{14}C]-oxidized-β-lactoglobulin, 4μM) whose degradation by protease La was not affected by DNA (Table 2.14).

2. The discovery that in two separate experiments ATPase activity was greater in the presence of DNA when this activity was measured in assays containing a substrate ([^{14}C]-β-lactamase, 2μM) whose digestion by protease La was

stimulated by DNA (Table 2.14). The stimulation of activity was small, and the statistical significance of it remains to be confirmed.

It is difficult to explain why such conflicting results concerning the effect of DNA on the proteolytic and ATPase activities of protease La *in vitro* have been obtained. These results are summarized in Table 3.1. It is also difficult to explain why there are conflicting reports over another property of protease La: is binding of ATP sufficient to support proteolytic activity or is hydrolysis of ATP required? A possible explanation for the inconsistent behaviour of protease La *in vitro* is that its proteolytic activity is tightly regulated *in vivo* to prevent extensive degradation of cellular protein. Possibly, regulation is lost *in vitro* because the concentrations of effectors (protein, ATP, ADP and DNA) and experimental conditions are inappropriate, or a factor required for regulation but not activity of protease La is lacking, causing abnormal activities to be detected. At present, it is impossible to differentiate normal from abnormal activities. As more work is carried out (approaches using physiological substrates such as λ*N* product seem very promising) the controversy over the activities of protease La *in vitro* may be solved. Until that time the physiological role of protease La cannot be ascertained.

3.3 CONCLUSION

During the course of this project protease La has been found *in vitro* to:

1. Cleave radiolabelled proteins at several sites in an ATP hydrolysis-dependent reaction.
2. Digest native proteins.
3. Show a high degree of specificity of degradation.

These findings, which have also been reported by others (Menon *et al.*, 1987; Maurizi, 1987), provide support for the proposal that protease La has a highly selective endopeptidase activity *in vivo*. This work and the recent discovery of another ATP-dependent protease (Ti) from *E. coli* (Katayama-Fujimura *et al.*, 1987; Hwang *et al.*, 1987), which has a different specificity to that of protease La (Hwang *et al.*, 1988), support the idea that ATP-dependent proteases play a significant role in conferring specificity to intracellular proteolysis in bacterial cells.

Unfortunately, the limited time available prevented the identification of the features of proteins that confer sensitivity to protease La, although it was possible to

TABLE 3.1 Effect of DNA on the Activities of Protease La

ACTIVITY	EFFECTS OF DNA ON PROTEASE La		
	Chung & Goldberg (1982)	Charette et al. (1984)	THIS THESIS
Proteolytic	DNA inhibits at low [^{3}H]-casein concentrations (<0.3μM); stimulates at higher concentrations.	DNA inhibits at low [^{3}H]-casein concentration (0.1μM); no effect at 2μM.	DNA stimulates ([^{14}C]-β-lactamase, 0.5-4μM), inhibits ([^{14}C]-α-lactalbumin, 0.5-5μM), or has no effect ([^{14}C]-casein, 3μM, and [^{14}C]-oxidized-β-lactoglobulin, 4μM).
ATPase measured in the presence of a protein substrate	DNA stimulates in the presence of [^{3}H]-casein (25μM).	DNA stimulates in the presence of [^{3}H]-casein (0.2μM).	DNA inhibits (in presence of [^{14}C]-α-lactalbumin 5μM), has no effect ([^{14}C]-oxidized-β-lactoglobulin, 4μM), or stimulates ([^{14}C]-β-lactamase, 2μM).
CONCLUSION	Single enzymic activity which is stimulated by DNA.	Two independent enzymic activities.	Single enzymic activity which is affected by DNA.

show that denaturation is not necessarily sufficient to convert a protein into a substrate for protease La. The approach that was originally intended to address this question was to characterize the degradation products of substrates of protease La (α-lactalbumin, casein, and histone preparations) in order to identify the sites of cleavage. This would have allowed comparison of the primary sequences flanking the peptide bonds cleaved. However, Maurizi (1987) adopted such an approach but found no obvious sequence similarities at the sites of cleavage of three substrates (oxidized insulin B chain, glucagon, and the λ*N* protein). The discovery that protease La has two binding sites for proteins (regulatory and catalytic; Waxman and Goldberg, 1986) suggests that protease La recognizes features of substrates other than the primary sequence at the cleavage site. In view of these findings, an alternative approach must be devised to address the problem. One possibility would be to select for mutants of a substrate of protease La which are no longer sensitive to cleavage. Sequence analysis of the mutants may then reveal residues or sequences recognized by protease La. The *rcsA* product is a possible candidate for such an approach because this protein has an increased half-life on *lon* mutants, and over-expression of the gene leads to a well defined phenotype (mucoidy; Torres-Cabassa and Gottesman, 1987).

Current understanding of the properties of protease La has been gained from *in vitro* studies. Such experiments may be misleading because of the non-physiological nature of conditions *in vitro*. The conflicting reports over the effect of DNA on the activities of protease La (Chung and Goldberg, 1982; Charette *et al.*, 1984; this thesis) and whether ATP hydrolysis is required for proteolytic activity (Chung and Goldberg, 1981; Charette *et al.*, 1981; Goldberg and Waxman, 1985; Maurizi, 1987; this thesis) may be a reflection of this. In particular, protease La may interact with other proteins *in vivo* (such as other heat shock proteins) which modulate the activities of the protease. It seems likely that until such complexity can be attained *in vitro*, or until detailed *in vivo* studies can supplement the current knowledge, a complete understanding of the role of protease La will not be realized.

CHAPTER 4

Materials and Methods

4.1 MATERIALS

4.1.1 Chemicals and Reagents

Chromatographic materials used were DEAE-cellulose DE-52, phosphocellulose P11 (from Whatman Ltd., Maidstone, Kent, England), Sephacryl S-300, Sephadex G-25F and G-75 (from Pharmacia G.B. Ltd., Milton Keynes, Bucks, England).

Paper for HVPE was purchased from Whatman Ltd.

[^{14}C]- and [^{3}H]-formaldehyde (10 and 72mCi mmol^{-1}, respectively) were obtained from New England Nuclear, Du Pont U.K. Ltd., Wedgwood Way, Stevenage, Herts, England.

[γ-^{32}P] ATP (3000Ci mmol^{-1}) was purchased from Amersham International plc., Amersham, Bucks, England.

Nitrocefin was a gift from Glaxo Group Research Ltd., Greenford, Middlesex, England.

Ficoll 400 (a non-ionic synthetic polymer of sucrose) was bought from Sigma Chemical Company Ltd., Poole, Dorset, England. Orange G and methyl green were purchased from BDH Ltd., Broom Road, Poole, Dorset, England.

4.1.2 Proteins

The following proteins were purchased from Sigma Chemical Company Ltd.: bovine α-lactalbumin, casein, pancreatic RNase, erythrocyte carbonic anhydrase and serum albumin; horse heart cytochrome *c*; hen egg albumin and lysozyme; soybean trypsin inhibitor; rabbit α-glycerophosphate dehydrogenase, phosphorylase b and glyceraldehyde-3-phosphate dehydrogenase; yeast enolase and 3-phosphoglycerate phosphokinase.

Other proteins used were:

1. Bovine β-lactoglobulin (Pentex Incorporated, Kanakee, Illinois 60901).
2. *S. aureus* P2 β-lactamase (gift from A. Coulson).
3. *S. aureus* PC1 and P54 β-lactamases (this thesis).
4. *E. coli* RTEM β-lactamase (gift from R.P. Ambler).
5. Chick erythrocyte histones (gift from K. Murray).

6. Bovine trypsin and chymotrypsin (Worthington Biochemical Corporation, Millipore U.K. Ltd., Harrow, Middlesex, England).

7. *Hin*dIII and *Bam*HI (Boehringer Corporation Ltd., Boehringer Mannheim House, Lewes, Sussex, England).

4.1.3 DNA

Plasmids pGC1 and pJMC40 were gifts from G. Cowan and A. Markovitz, respectively. Sonicated calf thymus DNA and λcI857 DNA were provided by D. Anderluzzi and J. Campbell, respectively.

4.1.4 Stock Solutions

Tris Buffers: Tris was adjusted to pH with HCl. Unless otherwise stated, the pH given is that at room temperature.

Ammonium Acetate Buffers: Acetic acid was adjusted to pH with 2M NH_3.

BICINE Buffers: BICINE was adjusted to pH with NaOH.

Borate Buffers: Boric acid was adjusted to pH with NaOH.

Phosphate Buffers: KH_2PO_4 and K_2HPO_4 of twice the desired molarity were mixed in the appropriate ratio to give the correct pH before diluting 2-fold.

TE: 10mM Tris; 1mM EDTA, adjusted to pH 8.0 with HCl.

4.1.5 Bacterial Strains

The bacterial strains used are described in Table 4.1.

4.1.6 Media

Dried media were purchased from Difco Laboratories Ltd., P.O. Box 14B, Central Avenue, East Molesey, Surrey, England. The following quantities are per litre of solution:

L-broth: 10g Difco Bacto-Tryptone; 5g Difco Bacto yeast extract; 10g NaCl; adjusted to pH 7.2 with NaOH.

L-agar: L-broth solidified with 15g Difco agar.

TABLE 4.1 Bacterial Strains

SPECIES	STRAIN	SOURCE	REFERENCE
Escherichia coli	NM522	G. Cowan	Gough and Murray (1983)
Staphylococcus aureus	PC1 (NCIB 11195)	J. Fleming	Ambler (1975)
S. aureus	P54	J. Fleming	Ambler (1979)

4.2 BACTERIAL TECHNIQUES

4.2.1 Maintenance of Strains

Short term storage of bacterial strains was achieved by streaking cultures on L-agar plates containing the appropriate antibiotic. The plates were incubated at 37°C until single colonies were observed and then the plates were maintained at 4°C. For long term storage at -20°C, 1ml of cultures were harvested by centrifugation (11,600g_n for 5min), resuspended in 1ml of 10mM $MgSO_4$, and 2ml of 80% (v:v) glycerol was added.

4.2.2 Growth of Strains

Liquid cultures were grown with aeration at 37°C to stationary phase in L-broth containing the appropriate antibiotic in 5ml volumes in 10ml bottles ("overnights") or in 500ml volumes in 2l Erlenmeyer flasks. Large scale cultures were grown in 50l Biotech fermenters at 37°C by T. Bruce. The stirrer speed was maintained at 250-300rpm and micro-aerophilic conditions were achieved by aerating with 1.5-3.5l of air min^{-1}. Cells were grown to late log phase and were harvested using an Alfa-Laval continuous flow centrifuge. Storage of cells was at -20°C until required.

4.2.3 Transformation of *E. coli* with pJMC40

Cells (NM522) were made competent for the uptake of DNA using a modification of the procedure of Mandel and Higa (1970).

A fresh overnight culture (5ml) was diluted 50-fold and grown, with aeration, at 37°C to an A_{650} of 0.7. The cells were harvested by centrifugation (2,000g_n for 5min) at 4°C and resuspended in half the original volume of ice-cold 100mM $CaCl_2$. After 30min on ice the cell suspension was centrifuged (2,000g_n for 5min) at 4°C and the cell pellet resuspended in one tenth of the original volume of ice-cold 100mM $CaCl_2$. The

competent cells were kept on ice for a minimum of 1h before use. The plasmid (200ng pJMC40) was diluted in 100µl SSC/$CaCl_2$ and was added to 200µl of competent cells. After 10-30min on ice the cells were "heat-shocked" at 37°C for 5min. The cells were incubated at 37°C for 1h in 1ml of L-broth to allow expression of tetracycline resistance. Samples of 10µl and 50µl were spread on L-agar plates containing tetracycline (20µg ml^{-1})and the plates were incubated at 37°C overnight.

SSC/$CaCl_2$: 6mM tri-sodium citrate; 60mM NaCl; 60mM $CaCl_2$.

4.3 DNA TECHNIQUES

4.3.1 Ethanol Precipitation of DNA

DNA was precipitated by the addition of 0.1 volumes of 3M sodium acetate and 2 volumes of ethanol. After incubation at -20°C for 30min the DNA was recovered by centrifugation at 8,000g_n for 10min. The DNA pellet was washed with 70% (v:v) ethanol, centrifuged (8,000g_n for 10min), and the resulting pellet was dried under vacuum. The DNA was dissolved in the appropriate volume of TE.

4.3.2 Determination of DNA Concentration

The absorbance at 260nm and 280nm of the DNA solution was measured. An A_{260} of 1 is equivalent to 50µg ml^{-1} DNA so the concentration of DNA in the sample (µg ml^{-1}) is given by A_{260} x 50. The ratio of A_{260}:A_{280} gives a measure of the purity of the DNA (Maniatis *et al.*, 1982) and DNA with values between 1.8 and 2.0 was used.

4.3.3 Small Scale Preparation of Plasmid DNA

(Ish-Horowicz and Burke, 1981)

The cells from 1.5ml of an overnight culture of NM522/pJMC40 were harvested by centrifugation at 11,600g_n for 5min, resuspended in 100µl of solution I, and incubated for 5min at room temperature. After addition of 200µl of solution II and gentle mixing, the mixture was kept on ice for 5min. Ice-cold solution III (150µl) was added, mixed gently, and the mixture was returned to ice for a further 5min. The resulting precipitate was removed by centrifugation (11,600g_n for 5min) and the DNA in the supernatant was precipitated by the addition of ethanol. The DNA pellet was dissolved in 50µl of TE, and aliquots of 15µl were used for restriction enzyme digests.

Solution I: 25mM Tris/HCl, pH 8.0; 10mM EDTA; 50mM glucose.

Solution II: 0.2M NaOH; 1% (w:v) SDS.

Solution III: 5M potassium acetate, pH 4.8.

4.3.4 Large Scale Preparation of Plasmid DNA

This method is based on that of Clewell and Helinski (1969). A fresh overnight culture of NM522/pJMC40 was diluted 100-fold in 150ml of L-broth containing tetracycline (20µg ml^{-1}) and grown overnight at 37°C, with aeration. The cells were harvested (6,500g_n for 10min), resuspended in 7ml of lysis solution, and left on ice for 5min. Addition of 14ml of alkaline SDS was followed by a 10min incubation on ice. After addition of 10.5ml of 3M potassium acetate pH 4.8, and a further 5min on ice, the precipitated protein, SDS, and chromosomal DNA were removed by centrifugation (6,500g_n for 10min at 4°C). To remove any remaining precipitate the supernatant was poured through glass wool. Plasmid DNA was precipitated by addition of 15ml of isopropanol, and recovered by centrifugation at 6,500g_n for 10min. The pellet was washed with 70% (v:v) ethanol, centrifuged (6,500g_n for 10min), and the resulting pellet dried under vacuum for 30min. The DNA was dissolved in TE (final volume 9.4ml), and CsCl (to 0.95g ml^{-1}) and ethidium bromide (to 0.6mg ml^{-1}) were added. The final density of the solution was 1.55g ml^{-1}. The solution was centrifuged at 90,000g_n for 48-60h at 18°C in 10ml "quick-seal" polyallomer tubes. After centrifugation two bands were visible under U.V. light: an upper band consisting of nicked and linearized plasmid DNA and fragmented chromosomal DNA; and a lower band consisting of supercoiled plasmid DNA. The lower band was removed using a 21 gauge hypodermic needle inserted through the side of the tube. Ethidium bromide was removed by four extractions with isopropanol saturated with NaCl-saturated TE. Two volumes of distilled H_2O were added to the aqueous phase before the DNA was precipitated with ethanol (without addition of sodium acetate). The DNA was dissolved in 500µl TE, and any residual protein was extracted twice with phenol equilibrated with TE. The DNA in the aqueous phase was ethanol precipitated and redissolved in 500µl of TE. The concentration of DNA was determined by measuring the absorbance at 260nm.

Lysis Solution: 25mM Tris/HCl, pH 8.0; 10mM EDTA, pH 8.0; 1% (w:v) glucose.

Alkaline SDS: 0.2M NaOH; 1% (w:v) SDS.

4.3.5 Restriction Endonuclease Digestion of DNA

Digestion of 0.5-1.0µg DNA was carried out in universal buffer (33mM Tris/acetate, pH 7.9; 10mM magnesium acetate; 5mM DTT; 1mg ml^{-1} BSA) with 2 units of restriction enzyme in a final volume of 20µl. Reaction mixtures were incubated at 37°C for 2h prior to analysis by agarose gel electrophoresis.

4.4 ELECTROPHORETIC TECHNIQUES

4.4.1 Agarose Gel Electrophoresis of DNA

Samples of DNA (0.2-0.5µg) were mixed with 5x Ficoll loading dye and loaded into wells of a 0.7% (w:v) agarose gel in 1x TBE. Electrophoresis in non-submerged gels was carried out at 1.5V cm^{-1} overnight; whereas submerged minigels were run at 11V cm^{-1} for 1h.

5x Ficoll Loading Dye: 20% (w:v) Ficoll 400 in H_2O, with bromophenol blue dye.

10x TBE: 890mM Tris; 890mM boric acid; 25mM EDTA.

4.4.2 Detection of DNA Fragments in Agarose Gels

The agarose gel was placed in a 1µg ml^{-1} solution of ethidium bromide for 20min and then washed for 20min in distilled H_2O. DNA bands in the agarose gel were visualized with a U.V. transilluminator and photographed using Ilford HP5 film.

4.4.3 SDS Polyacrylamide Gel Electrophoresis of Proteins

Two discontinuous buffer systems were used. Unless stated otherwise, SDS-PAGE was carried out with the buffer system of Laemmli (1970).

a Laemmli (1970)

Resolving gel mix (about 25ml) was poured between 20 x 16cm glass plates, separated by 1.5mm spacers. Distilled H_2O was carefully layered on top of the gel mix. When the gel had set, the H_2O was poured off and replaced by stacking gel mix. A comb was inserted into the stacking gel mix and when the stacking gel had set, the comb was removed to form sample wells. The glass plates and electrophoresis tank were clamped together and the assembly was filled with reservoir buffer. Samples were boiled for about 2min with sample loading buffer, before loading into wells using a microsyringe. Electrophoresis was carried out at 10mA (constant current) overnight.

Gradient resolving gels were prepared as above except that the concentrations of AMPS and TEMED were reduced 2-fold. Acrylamide mixes (15ml each) were prepared in a gradient former, and 15% (w:v) sucrose was added to the acrylamide mix containing the greater concentration of acrylamide.

Acrylamide Stock Solution: 30g acrylamide; 0.8g N,N'-methylene-bis-acrylamide; distilled H_2O to 100ml. Degassed and filtered through Whatman No. 1 paper, before storage in a dark bottle at 4°C.

Resolving Gel Buffer: 3.0M Tris/HCl, pH 8.8.

Stacking Gel Buffer: 0.5M Tris/HCl, pH 6.8.

Resolving Gel Mixes: See Table 4.2.

Stacking Gel Mix: 1.25ml acrylamide stock; 2.5ml stacking gel buffer; 0.1ml 10% (w:v) SDS; 0.5ml 1.5% (w:v) AMPS; 5.65ml distilled H_2O; 15µl TEMED.

10x Reservoir Buffer: 0.25M Tris; 1.92M glycine; 1% (w:v) SDS.

Sample Loading Buffer: 62.5mM Tris/HCl, pH 6.8; 2% (w:v) SDS; 5% (v:v) β-mercaptoethanol; 10% (w:v) sucrose; 0.1% (w:v) bromophenol blue.

TABLE 4.2 Composition of Resolving Gel Mixes for Polyacrylamide Gel Electrophoresis

COMPONENTS	FINAL CONCENTRATION OF ACRYLAMIDE IN RESOLVING GEL (%)						
	20	17.5	15	12.5	10	7.5	5
Acrylamide stock (ml)	20.0	17.5	15.0	12.5	10.0	7.5	5.0
Resolving Gel Buffer (ml)	3.75	3.75	3.75	3.75	3.75	3.75	3.75
10% SDS (ml)	0.3	0.3	0.3	0.3	0.3	0.3	0.3
1.5% AMPS (ml)	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Distilled H_2O (ml)	4.45	6.95	9.45	11.95	14.45	16.95	19.45
TEMED (µl)	15.0	15.0	15.0	15.0	15.0	15.0	15.0

b Anderson *et al.* (1983)

Gels were prepared as described in (a) except that the sample wells were washed out with distilled H_2O and then filled with sample loading buffer (without sucrose and bromophenol blue) before loading samples. Electrophoresis was carried out at a constant current of 35mA overnight.

Upper Reservoir Buffer (Cathode): 74mM Tris/HCl, pH 7.8; 0.1% (w:v) SDS.

Lower Reservoir Buffer (Anode): 200mM Tris/HCl, pH 7.8; 0.04% (w:v) SDS.

Stacking Gel Buffer: 200mM Tris/H_2SO_4, pH 7.8; 0.04% (w:v) SDS.

Resolving Gel Buffer: 200mM Tris/H_2SO_4, pH 7.8; 0.04% (w:v) SDS; 8M urea.

Stacking Gel Mix: 2.5g acrylamide; 0.63g N,N'-methylene-bis-acrylamide; stacking gel buffer to 100ml; 50mg AMPS; 50µl TEMED.

Resolving Gel Mix: 7.6g acrylamide; 0.4g N,N'-methylene-bis-acrylamide; resolving gel buffer to 100ml; 50mg AMPS; 50µl TEMED.

Sample Loading Buffer: 139mM Tris/acetate, pH 7.8; 1% (w:v) SDS; 20% (w:v) sucrose; 100mM (v:v) β-mercaptoethanol.

4.4.4 Detection of Polypeptides in Polyacrylamide Gels

Two methods of Coomassie Blue staining were used.

a In Acetic Acid/Methanol Solution

To fix the polypeptides and to remove the SDS (which can interfere with staining) the gel was immersed in fix for 30min. After immersing the gel in stain for 30min, the gel was placed in destain until the blue polypeptide bands were visible against a clear background (usually 16–20h). The destaining solution was changed when required.

Gel Fix: 10% (v:v) acetic acid; 20% (v:v) methanol.

Gel Stain: 7.5% (v:v) acetic acid; 50% (v:v) methanol; 0.25% (w:v) Coomassie Blue R250.

Gel Destain: 7% (v:v) acetic acid; 10% (v:v) methanol.

b In a Formaldehyde/Ethanol Solution (Steck *et al.*, 1980)

As an alternative to fixing by precipitation with a methanol/acetic acid solution, formaldehyde can be used to link the polypeptides to the gel matrix. Following immersion for 1h each in stain I and then stain II, the gel was placed in gel destain until the blue polypeptide bands were visible against a clear background (usually 16–20h). The gel destain was changed as required.

Stain I: 25% (v:v) ethanol; 14% (v:v) formaldehyde; 0.1% (w:v) Coomassie Blue R250.

Stain II: 25% (v:v) ethanol; 1% (v:v) formaldehyde; 0.12% (w:v) Coomassie Blue R250.

Gel Destain: 25% (v:v) ethanol; 1% (v:v) formaldehyde.

4.4.5 Detection of Radiolabelled Polypeptides in Polyacrylamide Gels

Gels containing radiolabelled polypeptides were stained with Coomassie Blue and then dried under vacuum at 80°C. The dried gels were exposed to Cronex X-ray film to produce an autoradiograph.

4.4.6 High Voltage Paper Electrophoresis of Peptides and Amino Acids

HVPE was carried out in Michl (1951) solvent-cooled tanks using the volatile pH 6.5 and 2.0 buffer systems described by Ambler (1963). Radioactive samples (dissolved in 10µl 0.1M NH_4OH) were spotted onto Whatman 3MM paper (peptide separations) or Whatman No. 1 paper (amino acid analysis). After electrophoresis the papers were dried in a warm, ventilated oven prior to staining and autoradiography.

a Electrophoresis at pH 6.5

The solvent system used was pyridine/acetic acid/H_2O (25:1:225 by volume) pH 6.5, and toluene was used as the coolant. "Wondermix" (5µl) was used as a marker (Milstein, 1966). Electrophoresis was performed at 3kV for about 1h and was monitored by following the migration of orange G.

b Electrophoresis at pH 2.0

The solvent system used was formic acid/acetic acid/H_2O (1:4:45 by volume) pH 2.0, and white spirit was used as the coolant. Amino acid mixtures R and T (5µl of each) were used as markers. Electrophoresis was performed at 3kV for about 1h and was monitored by following the migration of methyl green.

4.4.7 Two-Dimensional Separation of Amino Acids

(Waley and Watson, 1953)

A complex mixture of amino acids was resolved in the first dimension by HVPE at pH 2.0 and in the second dimension by descending paper chromatography for 12h, using the solvent mixture butan-2-ol/acetic acid/pyridine/H_2O (15:3:10:12 by volume).

4.4.8 Detection of Peptides and Amino Acids on Paper

(Toennies and Kolb, 1951)

Papers were immersed in a mixture of 0.1% (w:v) ninhydrin in acetone and 1% (v:v) (peptide mapping) or 4% (v:v) (amino acid analysis) 2,4,6 collidine in acetic acid (1:2, v:v). Colours were developed by allowing the paper to dry and then heating at 105°C for 30-120s. Free amino acids give a range of colours (Table 4.3) which fade to blue after 6-12h, whereas peptides generally develop as blue spots.

TABLE 4.3 Colours of Amino Acids Stained With a Ninhydrin/Collidine Mixture

AMINO ACID	COLOUR
Lys	blue
Arg	blue
His	brown
Gly	red/brown
Ala	blue
Val	blue
Ile	blue
Leu	blue
Ser	green
Thr	blue
Pro	yellow
Glu	blue
Phe	brown
Tyr	brown
Asp	turquoise
Mes [a]	blue
Cya [a]	blue

a. Mes: methionine sulphone; Cya: cysteic acid

4.4.9 Detection of Radiolabelled Peptides and Amino Acids on Paper

Papers were stained with a ninhydrin/collidine mixture and were then exposed to Cronex X-ray film to produce an autoradiograph.

4.5 PROTEIN TECHNIQUES

4.5.1 Performic Acid Oxidation of β-Lactoglobulin

(Hirs, 1956)

A sample of β-lactoglobulin (20mg) was incubated at 4°C for 1h with 2ml of freshly prepared performic acid (formic acid and 30% (v:v) hydrogen peroxide, 19:1 by volume, left for 2h at room temperature). The mixture was diluted 20-fold with distilled H_2O prior to lyophilization.

4.5.2 Treatment to Remove TCA from Samples

(Harris and Hindley, 1965)

A dried sample was resuspended in 1ml of acetone. The sample was centrifuged (11,600g_n for 3min) and the supernatant discarded. The precipitate was resuspended

in 1ml acetone, centrifuged (11,600g_n for 3min), and the supernatant discarded. This treatment was repeated with diethyl ether. After the final wash with diethyl ether, the sample was dissolved in 50µl of distilled H_2O and dried under vacuum.

4.5.3 Column Chromatography

Columns were normally pumped with a LKB microperpex peristaltic pump at the flow rates shown in Table 4.4. If the buffer used during column chromatography contained glycerol then the flow rate was decreased by a factor of two.

TABLE 4.4 Flow Rates Used During Column Chromatography

CHROMATOGRAPHIC METHOD	FLOW RATE (cm h^{-1})	
	4°C	RT [a]
Ion-exchange	15-20	30-40
Gel filtration:		
Sephadex G-25F & G-75	2-5	4-10
Sephacryl S-300	10-15	20-30

a. RT: room temperature.

4.5.4 Determination of Protein Concentration

(Lowry *et al.*, 1951)

Reagent A (3ml) was added to each 0.5ml sample. After 10min, 0.3ml reagent B was added, and the sample was mixed thoroughly. Samples were incubated at room temperature for 30min before measuring the absorbance at 750nm. The method was standardized against 25-400µg serum albumin.

Reagent A: 100ml 2% (w:v) Na_2CO_3 in 0.1M NaOH; 2ml 1% (w:v) NaK tartrate; 0.5ml 2% (w:v) $CuSO_4.5H_2O$.

Reagent B: Folin-Ciocalteau reagent diluted 10-fold.

4.5.5 Determination of Acid-Soluble Radioactivity

A modification of the method of Goldberg *et al.* (1982) was used.

Ice-cold BSA (final concentration 2mg ml^{-1}) and TCA (final concentration 10% w:v) were added to the sample. After incubation on ice for at least 30min, the sample was centrifuged (11,600g_n for 10min) at room temperature. A portion (normally 0.4ml) of the supernatant was pipetted into a 5ml scintillation vial insert and 4ml of scintillation fluid (333ml Triton X-100; 667ml toluene; 4.0g PPO; 0.1g dimethyl POPOP) was added. The scintillation vial was shaken vigorously before counting in a Beckman LS7000 liquid scintillation counter.

4.5.6 Radiolabelling of Proteins by Reductive Methylation

Two methods of labelling were used.

a With Sodium Borohydride (Rice and Means, 1971)

A sample of protein (0.1mg) was dissolved in 100µl of 200mM sodium borate, pH 9.0. To the solution kept on ice, 10µl of [^{14}C]-formaldehyde (10mCi $mmol^{-1}$, 40µmol ml^{-1}) were added. This was followed in 30s by four 2µl sequential additions of sodium borohydride (5mg ml^{-1}). To ensure complete reduction, an additional 10µl of sodium borohydride was added after 1min. Low molecular weight components of the reaction mixture were removed by exhaustive dialysis at 4°C against 50mM Tris/HCl, pH 8.0.

b With Sodium Cyanoborohydride (Jentoft and Dearborn, 1979)

A sample of protein containing 1.5µmol lysine residues was incubated with either 3µmol [^{14}C]-formaldehyde (10mCi $mmol^{-1}$) or 3µmol [^{3}H]-formaldehyde (72mCi $mmol^{-1}$), and 30µmol sodium cyanoborohydride (1mmol ml^{-1}) in 200mM sodium borate, pH 9.5. The reaction mixture (final volume 1ml) was incubated overnight at room temperature. Low molecular weight components of the reaction mixture were removed by exhaustive dialysis at 4°C against 50mM Tris/HCl, pH 8.0.

4.5.7 Acid Hydrolysis of Radiolabelled Proteins

Samples of radiolabelled proteins (1-50µg) were dried under vacuum, and 100µl 6M HCl was added. After incubation at 105°C overnight, the hydrolysate was dried in a vacuum desiccator over NaOH.

4.6 ENZYMIC ASSAYS

4.6.1 Assay of ATP-Dependent Proteolytic Activity

A modification of the method of Goldberg *et al.* (1982) was used.

Routinely, samples were incubated in the presence and absence of ATP (final concentration 3mM), with 5-20µg [^{14}C]-methyl protein (0.5-2 x 10^7cpm mg^{-1}) in assay buffer (50mM Tris/HCl, pH 8.0; 25mM $MgCl_2$) in a final volume of 500µl. After incubation at 37°C for 1-3h, acid-soluble radioactivity was determined.

4.6.2 Assay of ATPase Activity

A modification of the method of Eskin and Linn (1972) was used.

Routinely, assays contained 25mM Tris/HCl, pH 8.0; 25mM $MgCl_2$; 0.35µg protease La and 0.5mM [γ-^{32}P] ATP (10-25cpm $pmol^{-1}$) in a final volume of 30µl. The ATPase activity of protease La was also measured in the presence of varying concentrations of single- and double-stranded DNA (ssDNA was sonicated calf thymus DNA boiled for 10min then placed in ice; dsDNA was pGC1) and varying concentrations of radiolabelled proteins. After incubation at 37°C for 1-3h, the assays were diluted to 0.3ml with distilled H_2O before addition of BSA to a final concentration of 25mg ml^{-1}. Following addition of 0.1ml each of 1M HCl, 1M potassium phosphate and 20% (w:v) acid washed activated charcoal, the suspension was mixed thoroughly and incubated on ice for 5 min. The suspension was centrifuged (11,600g for 5 min) and 500µl of the supernatant was counted (Cerenkov) in a Beckman LS 7000 liquid scintillation counter.

4.6.3 Assay of β-Lactamase Activity

Two methods of assay were used.

a Quantitative Assay Using Benzylpenicillin (Waley, 1974)

Assays contained 3ml of 100mM potassium phosphate buffer, pH 7.0; 5µl of 35mg ml^{-1} benzylpenicillin and a suitable volume of sample. The decrease in absorbance at 232nm was followed after addition of sample. β-lactamase activity was expressed as µmol benzylpenicillin hydrolysed h^{-1} using a molar extinction coefficient of 940.

b Qualitative Assay Using Nitrocefin (O'Callaghan *et al.*, 1972)

Spot tests for β-lactamase activity were performed by mixing 10µl of sample with 10µl of nitrocefin solution in a well of a microtitre plate. β-lactamase activity was indicated by a colour change (from orange to purple).

Nitrocefin Solution: 5mg nitrocefin, dissolved in 0.5ml dimethylsulphoxide, to which 9.5ml 0.1M potassium phosphate buffer, pH 7.0 was added. The solution was stored in the dark at 4°C for up to two weeks.

4.7 PURIFICATION PROTOCOLS

4.7.1 Preparation of an Extract of NM522/pJMC40

Cells were resuspended in buffer A or B (volume equal to twice the wet weight of cells) and disrupted by sonication at 4°C with a MSE 100W Ultrasonic Disintegrator at about 6μM peak to peak using 1min bursts, with a 1min interval between bursts, until the cells had lysed (normally for 50g of cells, 8 x 1min bursts were sufficient). The lysed cells were centrifuged (48,000g_n for 45min) at 4°C to remove the cell debris. If buffer A was used, the supernatant was dialysed exhaustively at 4°C against buffer C.

Buffer A: 50mM Tris/HCl, pH 7.8 at 4°C; 200mM KCl; 10mM β-mercaptoethanol; 20% (v:v) glycerol.

Buffer B: 100mM KH_2PO_4/K_2HPO_4, pH 6.5; 10mM β-mercaptoethanol; 1mM EDTA; 20% (v:v) glycerol.

Buffer C: 10mM Tris/HCl, pH 7.8 at 4°C; 5mM β-mercaptoethanol; 20% (v:v) glycerol.

4.7.2 Purification of the *E. coli* ATP-Dependent Protease La

(Zehnbauer *et al.*, 1981; as modified by Goldberg *et al.*, 1982.)

An extract of 50g NM522/pJMC40 was prepared as described in Section 4.7.1 using buffer B. The following operations were performed at 4°C unless stated otherwise. The cell extract (20mg ml^{-1}) was loaded onto a phosphocellulose (P11) column (20 x 2.8cm). The P11 column was washed with 2 column volumes of buffer B and eluted with a 100mM to 400mM linear phosphate gradient comprised of equal volumes of buffer B and buffer D. Active fractions were pooled and dialysed against buffer E and loaded onto a DEAE-cellulose (DE-52) column (10 x 2.5cm). The DE-52 column was washed with buffer E (until low A_{280}) and eluted sequentially with buffer E containing 100mM NaCl and buffer E containing 300mM NaCl. Active fractions were pooled, concentrated by ultracentrifugation (Amicon Centricon-30 system) at room temperature and loaded onto a Sephacryl S-300 column (60 x 1.6cm) equilibrated with buffer F. Active fractions were pooled and stored at -70°C after addition of EDTA (final concentration 2mM) and DTT (final concentration 2mM).

Buffer B: 100mM KH_2PO_4/K_2HPO_4, pH 6.5; 10mM β-mercaptoethanol; 1mM EDTA; 20% (v:v) glycerol.

Buffer D: 400mM KH_2PO_4/K_2HPO_4, pH 6.5; 10mM β-mercaptoethanol; 1mM EDTA; 20% (v:v) glycerol.

Buffer E: 10mM Tris/HCl, pH 7.1; 10mM β-mercaptoethanol; 20mM NaCl; 20% (v:v) glycerol.

Buffer F: 50mM Tris/HCl, pH 7.8; 5mM $MgCl_2$; 100mM NaCl; 20% (v:v) glycerol.

4.7.3 Purification of *S. aureus* PC1 β-Lactamase

(Richmond, 1963; as modified by Ambler and Meadway 1969)

The supernatant of a 50l culture of *S. aureus* PC1 grown to late log phase was provided by T. Bruce. When sufficient phosphocellulose (P11, swollen and de-fined in distilled H_2O) had been added to adsorb about 90% of the β-lactamase, the culture supernatant was decanted. After washing with about 5l of 10mM ammonium acetate, pH 7.0 (buffer A), the phosphocellulose was poured into a column (20 x 9cm) and allowed to pack under gravity. The column was washed exhaustively with buffer A until negligible amounts of protein were eluted (low A_{280}). The β-lactamase was eluted with a 75% saturated solution of ammonium sulphate in buffer A. Active fractions were pooled and 40ml aliquots were loaded onto a Sephadex G-75 column (80 x 5cm) equilibrated with 100mM ammonium acetate, pH 7.0 (buffer B). Fractions containing β-lactamase were pooled and dialysed exhaustively at 4°C against distilled H_2O before lyophilization.

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