GENETIC AND BIOCHEMICAL STUDIES OF MICROBIAL PEPTIDASE ENZYMES

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Abstract

Investigations were undertaken on the microbial peptidase enzymes catalysing hydrolysis of L-α-aspartyl peptides, particularly those peptidases synthesised by the bacterium Escherichia coli K12. Bacterial strains, though not all fungal strains tested, used peptides as sources of required nutrients. All microbial strains, however, possessed aspartyl peptidase activities, demonstrable using a PAGE based activity stain. E. coli K12 produced 2 bands of aspartyl peptidase activity, mobilities 0.3 and 0.5 on 7.5% polyacrylamide gels, the former corresponding to the previously described peptidase B, and the latter an aspartyl peptide specific activity previously undescribed in this organism, designated peptidase E. Growth tests on peptidase recombinants indicated a third enzyme, peptidase Q, a prolyl peptidase, catalysed hydrolysis of Asp-Pro. Peptidase B and E activities, along with an Acinetobacter calcoaceticus aspartyl-β-napthylamide (ANA) hydrolysing peptidase were partially purified by ammonium sulphate fractionnation, ion-exchange chromatography and lastly gel filtration, which was used to estimate molecular weights (M_r) at 230kd, 35kd and 480kd respectively. These peptidases catalysed hydrolysis of N-terminal aspartyl peptides but not C-terminal aspartyl, N-terminal asparaginyl or glutamyl peptides, and all showed optimal activity at pH 7.8 with both peptidase E and ANA hydrolysing activities remaining high at pH 10. Sulphydryl and serine protease inhibitors did not significantly affect peptidase activities whereas the metal-ion chelator EDTA inhibited peptidase B and ANA hydrolysing activities though not peptidase E. Isolation of E. coli K12 mutants lacking aspartyl peptidase activity or unable to utilise aspartyl peptides proved impossible, however the gene encoding peptidase B was mapped at 57.5 minutes on the genome by interrupted mating and P1 transduction. Levels of peptidase B and E activities were higher in stationary phase than exponential cells, and higher in glycerol grown than glucose grown cells, but activity levels were unaffected by inclusion of aspartyl peptides in growth media. No synthesis of the aspartyl dipeptide sweetener, aspartame (Asp-PheOMe), was observed when whole bacterial cells, crude cell extracts and partially purified peptidases were used as catalysts for sweetener synthesis under various experimental conditions.

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Abbreviations

ANA	Aspartyl-β-napthylamide
ATP	Adenosine tri-phosphate
CBZ-Asp	Carboxybenzoyl-aspartate
dH_2O	distilled water
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DTNB	5,5'-Dithiobis (2-nitrobenzoic acid)
EDTA	Ethylenediaminetetraacetic acid
HPLC	High Performance Liquid Chromatography
NTG	N-methyl-N'-nitro-N-nitrosoguanidine
OPA	O-pthalaldehyde
PAGE	Polyacrylamide Gel Electrophoresis
PCMB	Para Polychloromercuribenzoate
PMSF	Phenylmethylsulphonyl chloride
PUVA	Psoralen-Ultra violet light A
RNA	Ribonucleic acid
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-tetramethylenediamine
TLC	Thin Layer Chromatography

CHAPTER 1: GENERAL INTRODUCTION

1.1 The Terminology and Classification of Peptidase Enzymes

Peptidase enzymes have been identified in all biological systems studied; in plant, mammalian and microbial systems (Zuber, 1964; Hermsdorf & Simmonds, 1980; McDonald & Barrett, 1986). The literature on these peptidases however, contains many terms which would be unfamiliar and confusing to those uninitiated in this field. There therefore follows a brief survey of some of the terminology and of the classification of these enzymes.

Enzymes are biological catalysts which increase the rate of attainment of equilibrium in biochemical reactions. Peptidase enzymes form one of 2 sub-groups (the other referred to as the proteinases) within a class of enzymes, the proteases, which catalyse the H_2O-mediated cleavage (hydrolysis) of peptide bonds (IUB/IUPAC Nomenclature Committee 1978). Peptide bonds are generated by condensation reactions between amino acid molecules, and amino acid polymers (peptides), containing many peptide bonds have been found in all biological systems. These peptides range in size from 2 to many hundreds of amino acid monomer units (Lehninger, 1975).

It is the smaller peptide residues containing from 1-5 amino acid molecules which in general form the substrates for the microbial peptidase enzymes discussed in this work. These peptidase enzymes exhibit only exopeptidase activity, the ability

to degrade peptide chains from their ends, in contrast to the proteinases which all exhibit endopeptidase activity, the ability to hydrolyse peptide bonds within a long peptide chain. A protease is classified as a proteinase if it exhibits a significant degree of endopeptidase activity, regardless of whether it exhibits exopeptidase activity (McDonald & Barrett, 1986).

The peptidase enzymes themselves are divided into classes according to their specificity (IUB/IUPAC Nomenclature Committee 1978). Those peptidases hydrolysing single amino acid residues from the N-terminus (see below) of the peptide chain are classified as aminopeptidases, those hydrolysing single residues from the C-terminus (see below) are classified as carboxypeptidases. Dipeptidases hydrolyse only dipeptide molecules containing 2 amino acid residues. Those peptidases which split off dipeptide units from the N-terminus or C-terminus of the peptide chain are classified as dipeptidyl peptidases and peptidyl dipeptidases respectively. Further sub-division within the aminopeptidase class on the basis of subtrate size gives a group of tripeptidase enzymes. Carboxypeptidases are sub-divided on the basis of enzyme mechanisms. One class displays maximum activity in the acid range and is inhibited by the substitution of a serine residue by organic fluorophosphates (serine carboxypeptidases) and the second class requires divalent cations for activity (metallocarboxypeptidases). A new class of peptidases has also been recently defined. These 'omega

peptidases' are capable of removing terminal residues that lack either a free N-terminal or C-terminal residue (McDonald and Barrett, 1986).

The studies carried out in this thesis focused mainly on the isolation and characterisation of peptidase enzymes catalysing the hydrolysis of aspartyl dipeptides. Most of this work was performed using the well-studied bacterium Escherichia coli K12, the dipeptidases and aminopeptidases of which are well characterised. These enzymes therefore proved most useful and relevant in comparative studies on isolated aspartyl-peptide hydrolysing enzymes.

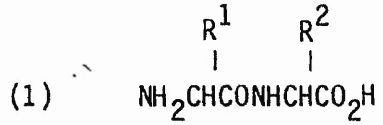
In order to gain a more thorough understanding of peptidase enzymes, in particular in terms of their role in microbial systems, an appreciation of the structure, function and biochemistry of their peptide substrates is required as well as a knowledge of the means by which microbial systems generate these peptides. These areas are covered in the following section.

1.2 The Biochemistry, Biosynthesis and Microbial Metabolism of Peptides

It is difficult to overstate the importance of peptides in biological systems. They occur in all living organisms and are considered fundamental to life itself. The high molecular mass peptides (>10,000d) include soluble proteins such as the enzymes on which nearly all biochemical processes depend and through which genes exercise their control over metabolism (peptidase enzymes themselves are in fact high molecular weight peptides!), as well as the fibrous proteins involved in maintaining cell structure. Peptides containing in the region of 8-30 residues include the siderophores such as ferrichrome, involved in the chelation and cellular uptake of iron in various fungal strains (Bågg & Neilands, 1987), as well as peptide antibiotics such as gramicidin S, an anti-bacterial ionophore secreted by the Gram-positive bacterium Bacillus brevis, that increases the permeability of target membranes to specific ions (Katz & Demain, 1977). The smaller peptides, which form the substrates for the peptidase enzymes, in general exist only transiently in microbial cells and are rapidly degraded to their amino acid constituents (Payne & Bell, 1979, Yen et al., 1980a).

The biochemistry of peptides has been well studied (Lehninger, 1975). Peptides are composed of α-amino acids joined together byamide (peptide) bonds, (1). The simplest of these compounds ($R^1 = R^2 = H$) possesses only 2 amino acid units per

molecules, whereas more complex examples incorporate a large number of amino acids and, in terms of molecular mass can be as much as ten thousand times larger. Peptides of relatively low molecular mass (M_r < 10,000) are called polypeptides, the higher molecular mass materials, proteins. Small polypeptides, sometimes referred to as oligopeptides are given names which indicate the number of amino acid units, dipeptides possess 2 such units, tripeptides three, tetrapeptides four, and so on.



(1)

The amino acid sequences of peptides are conventionally written with the terminal α-amino group to the left and the peptides named as acyl-substituents of the carboxy-terminal amino acid residue. Thus, the simplest peptide referred to above (R^1 = R^2 = H) is glycylglycine, usually abbreviated using the 3 letter amino acid symbols to Gly-Gly. Any of the 20 different naturally occurring amino acids can be found at any position in a peptide, polypeptide or protein and this positional lack of selectivity provides the basis for the immense structural and functional diversity of these molecules. Even for a relatively tiny tetrapeptide there exist a possible 20^4 = 160,000 different amino acid sequences, providing an immense number of potential substrates for peptidase enzymes.

In living systems all peptides derived from proteins

consist of L-form stereoisomers of amino acids. On relatively short peptides, the total observed optical activity is approximately an additive function of the optical activities of the component amino acids. D-form amino acids are found in microbial cell walls and peptide antibiotics. Some non-protein derived synthetic peptides substrates, for example amino acid β-napthylamides, are used in bio-chemical assays of peptidase enzyme activity due to their chromogenic properties, and though some reservations have been expressed about this practice due to doubt about their occurrence as natural substrates the relative ease of their assays ensures their continued use. A multitude of other novel peptide and peptide derivatives have been chemically synthesised but of these only the dipeptide methyl-ester aspartame will be further mentioned in this study.

As with amino acids, the presence of free amino acid carboxyl molecules tends to give rise to dipolar ions, or zwitterions. This property is reflected in the amphoteric nature of peptides which can behave as either proton donating acids or proton accepting bases. None of the α-carboxyl groups and none of the α-amino groups that are combined in peptide linkages can ionise in the pH range 0-14 and so the acid-base behaviour of peptides is contributed by the free α-amino group of the N-terminal residue, the free α-carboxyl group of the C-terminal group and the α-groups in intermediate positions which can ionise. The acid-base titration curves of short peptides are very similar to those of free α-amino acids and the predominant

ionic species at different stages in the titration curves of peptides are comparable to those for free amino acids. Like amino acids, peptides also have a characteristic isoelectric pH at which the molecule has no net charge. The presence of the free N-terminal amino acid groups of peptides means that peptides undergo the same kinds of chemical reactions as those given by the amino groups of free amino acids. This property is made use of in the work presented here, in the form of ninhydrin and O-pthalaldehyde (OPA) colorimetric and fluorescence based chemical assays (ninhydrin and OPA react quantitatively with N-terminal residues from both amino acids and peptides).

As far as is known, with the sole exception of peptide residues contained within cell wall peptidoglycan, those small peptides, hydrolysable by peptidase enzymes, are generated in the microbial cell, either as intermediates formed during the processing or degradation of high molecular mass polypeptides and proteins (Wickner, 1980, Yen et al., 1980b), or through the uptake into the cell of externally supplied peptides (Payne, 1976). In addition small peptide chains are thought to be generated as mistranslation products during the biosynthesis of polypeptides and proteins (Menninger, 1976, 1978; Manley, 1978). The process of nucleic acid mediated polypeptide/protein biosynthesis has been widely reviewed (Lehninger, 1975; Watson, 1978; Hershey, 1987) and is briefly summarised here as it forms an important part of the background in which peptidases function.

Nucleic acid mediated polypeptide/protein synthesis requires a complex apparatus which translates information encoded in nucleic acid sequences into amino acid sequences. Amino acids are activated by formation of a high energy ester bond to a specific 'transfer' RNA (t-RNA) molecule in an energy consuming, adenosine triphosphate (ATP) requiring reaction catalysed by an 'aminoacyl-tRNA synthetase' enzyme. The product aminoacyl-tRNA's are linearly ordered by interaction with a template, the messenger RNA (mRNA); in effect, the tRNA molecule acts as an adaptor between the amino acid and the mRNA. These actions occur on the surface of cellular organelles called ribosomes.

The polymerisation process is divided into 3 phases, initiation, elongation and termination. During initiation, a unique initiator aminoacyl-tRNA binds at a precise region of the mRNA thereby specifying the phase in which the mRNA is translated. Elongation is a cyclic process whereby subsequent aminoacyl-tRNA molecules bind to the ribosome as dictated by the mRNA. In each case a peptide bond is formed by transfer of the aminoacyl (or peptidyl) moiety to the amino group of the incoming aminoacyl-tRNA and the ribosome progresses down the mRNA. During termination, the mRNA signals the binding of a factor that results in the hydrolysis of the completed peptidyl-tRNA which may in the case of premature termination cause the release of small peptide fragments. Polymerisation on the ribosomes is promoted by soluble protein factors specific for the 3 phases of synthesis. Thus the peptide chain grows from its N-terminus

towards its C-terminus and the sequence of amino acids is colinear with the nucleic acid sequence in the mRNA. Energy is consumed during the charging of the amino acids to their tRNA's and during the polymerisation process, representing a large investment by the cell in the biosynthesis process. In cells of the Gram-negative bacterium Escherichia coli K12 it has been estimated that up to 90% of biosynthetic energy may be used in protein biosynthesis.

Peptide antibiotics and cell wall peptides containing D-amino acid residues appear to be synthesized enzymically without the participation of mRNA templates or ribosomes. Particularly well studied is the enzymic synthesis by cells of the bacterium Bacillus brevis, of the cyclic peptide antibiotic gramicidin S which has 10 amino acid residues. This peptide contains residues of L-ornithine and D-phenylalanine which are not found in proteins. Its synthesis is brought about by a soluble complex of 2 enzymes together called gramicidin synthetase (Katz & Demain, 1977). Enzyme I catalyses the formation from the incoming amino acid of an enzyme bound acyl-adenosine monophosphate (Acyl-AMP) derivative which is then transferred to an -SH group on the enzyme. Enzyme I acts in this way to activate in the correct sequence, the four L-amino acids required for synthesis of Gramicidin S. Enzyme II activates L-phenylalanine in a similar manner and then promotes the inversion of the α-carbon atom to yield the bound D-stereoisomer. The amino acid unit esterified to enzyme I is

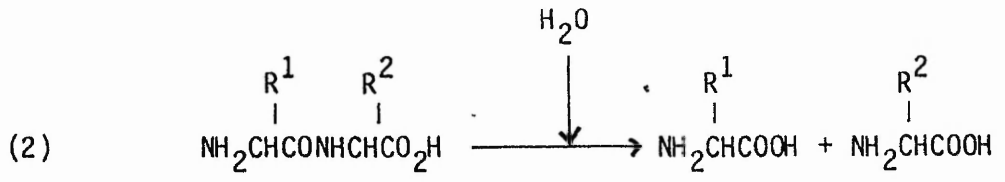
then attached to enzyme II to form a dipeptide. Successive activated L-amino residues are transferred to the growing peptide chain until a pentapeptidyl residue is produced. Two such peptides are then joined to yield the complete cyclic decapeptide.

The cell wall peptidoglycan of bacteria is synthesised in 3 stages, the first involving production of N-acetylmuramylpentapeptide, a recurring unit of the peptidoglycan backbone structure (Rogers et al., 1980). The pentapeptide part of this molecule is synthesised by sequential addition of 3 amino acids to the carboxyl group of a muramic acid residue followed by addition of a pre-synthesised dipeptide. The addition of each amino acid and the dipeptide to the peptide chain is catalysed by a specific ligase enzyme and involves the concommitant hydrolysis of ATP. The order of the amino acids in the peptide is determined by the specificity of the ligases towards their respective substrates.

The final destinations of the peptides, polypeptides and proteins synthesised by microbial cells are many. The iron chelating siderophores produced by fungal cells, the peptide antibiotics produced by the Bacilli, and many of the enzymes synthesised by both fungal and bacterial strains (in particular Gram-positive bacteria) are secreted outside of the cell into the extracellular environment. Such secretion may require processing of nascent polypeptide chains which itself may result in the generation of small peptides. Some proteins may be incorporated

into the outer cellular membranes of Gram-negative bacteria, for example the porins synthesised by Escherichia coli K12 (Nikaido & Vaara, 1985). In this same bacterial strain, permease proteins involved in the uptake of a variety of substrates, are located in the cytoplasmic inner membrane (Costerton et al., 1974), and nucleotidase, phosphatase, penicillinase and amino acid binding proteins are found in the periplasmic space between the inner and outer membranes (Neu & Heppel, 1965). Enzymes such as DNA polymerase, β-galactosidase and peptidase enzymes themselves have been localised in the cytoplasm of E. coli K12 (Neu & Heppel, 1965; McAman & Villarejo, 1982).

The intracellular proteins of bacterial and fungal strains are subject to degradation and turnover (Mandelstam, 1954; Goldberg & Dice, 1974). This turnover allows cells to remove abnormal proteins and provides a means by which the cell can synthesise new proteins required for different environmental conditions. The cellular proteins vary in their stability, and their rate of degradation is dependent on a number of factors including the phase of cell growth and prevailing environmental conditions. The degradation of these proteins is mediated in the first instance by proteinase enzymes which generate smaller peptides which are themselves hydrolysed in reactions catalysed by peptidase enzymes(2). The amino acids produced



from these hydrolyses are then available for use by the cell in the synthesis of new proteins, or after further breakdown as sources of carbon, nitrogen or sulphur.

It has been shown therefore that microbial proteins, polypeptides and peptides exist within a dynamic cellular framework of synthesis and degradation. The regulation of these mechanisms enables the cell to produce certain proteins only when required, enabling economic management of the cellular regime. The generation of new proteins may be facilitated by the uptake of peptides used as bacterial growth substrates. The following section considers the evidence for the use of peptides in microbial nutrition.

1.3 The Utilisation of Peptides in Microbial Nutrition

It has long been known that small peptides (1-5 amino acid residues) could be of great nutritional value for many types of bacteria and fungi (Payne, 1980), and as a result commercial peptones are routinely incorporated into a variety of growth media. The microbial utilisation of these peptides requires their hydrolysis by peptidase enzymes to their constituent amino acids. Detailed studies have shown that free amino acid residues and amino residues contained within peptide molecules, may give varying growth responses when supplied to microbes as nutritional sources. These effects are measured by analysis of microbial growth rates and growth yields. They are most readily observed and have been most closely studied using bacterial and fungal strains with auxotrophic requirements for particular amino acids.

Much of this work has been carried out using the Gram-negative bacterium *E. coli*, the amino acid auxotrophs of which can use a wide range of small peptide substrates for cell growth. Such substrates include the aspartyl peptides, a group of peptides of particular interest in this study. The responses to peptides and free amino acids have been found to be identical for phenylalanine and proline auxotrophs (Simmonds & Fruton 1949), lysine auxotrophs (Simmonds *et al.*, 1976) and virtually all other amino acid auxotrophs tested (Fruton & Simmonds, 1950). These studies used mainly di-, and tri-peptide substrates. Such a similar response to different amino acids and peptide sources

is not the rule amongst the bacterial and fungal strains used in growth tests and is presumably a consequence of the non-fastidious nature of E. coli, and the presence of highly efficient processes for the uptake and hydrolysis of peptides. Leucyl peptides form a unique group within E. coli serving not only as growth factors but also as growth inhibitors (Meisler & Simmonds, 1963; Simmonds, 1970). Growth inhibition results from the bacteriostatic action of the peptide and peptidase mediated hydrolysis relieves the bacteriostatic effect and provides the leucine required for growth of a leucine auxotroph. The closely related enteric Gram-negative bacterium Salmonella typhimurium can also utilise a wide range of peptide substrates for growth including aspartyl peptides (Miller & Mackinnon, 1974; Kirsch et al., 1978; Carter & Miller, 1984).

With Pseudomonas species a variety of responses have been observed. Amino acid auxotrophs (his, trp, thr or met mutants) of P. putida grew on a variety of di- and tri-peptides up to twice as slowly as with free amino acids. Final growth yields were 5-10 times greater when P. putida his^- was grown on histidyl di-, or tripeptides rather than on free histidine because the histidyl residues were protected from catabolism by the enzyme L-histidine ammonia lyase (Cascieri & Mallette, 1976a). In P. maltophilia (Cascieri & Mallette 1976b) and P. aeruginosa (Miller & Becker, 1978) methionyl peptides would satisfy the methionine requirement and although growth rates were all broadly similar, a 2-fold variation in growth yield was noted amongst a

range of methionyl peptides. These effects may occur because peptide uptake and/or hydrolysis can be the rate limiting process for overall growth.

Multiple auxotrophic bacterial species such as the Gram-positive Streptococci and Lactobacilli commonly exhibit quite different responses to peptides and amino acids. Peptides may be superior to amino acids in stimulating growth rates when the medium contains many required amino acids and competition for uptake between them causes a growth limitation; growth yields are often greater on peptides because the residues are protected from catabolism. Bacterioides species are reportedly able to grow on peptides but not on free amino acids (Pittman & Bryant, 1964; Pittman et al., 1967) although other reports indicate that free amino acids can stimulate the same response as with peptides (Wright, 1967; Lev, 1977).

With the exception of yeasts, studies of peptide utilisation in fungi, have focused on the Ascomycete fungus Neurospora crassa. These studies have provided a clearer understanding of the overall capability of this fungal organism to use proteins from the environment as sources of required metabolites. In an early attempt to evaluate the ability of Neurospora to grow on peptides as a source of some required amino acids, mutants auxotrophic for four different amino acids were screened for their ability to grow on a wide spectrum of peptides (Wolfinbarger & Marzluf, 1974). With a single lysine auxotroph,

poor growth on lysine containing dipeptides was observed while the tripeptide lys-lys-lys gave good growth. Inclusion of excess arginine so as to prevent transport of free lysine precluded growth on all peptides except tri-lysine. Methionine auxotrophs grew to various extents on all methionine peptides tested, as did both histidine and leucine auxotrophs, but blocking of amino acid transport systems prevented growth on dipeptides suggesting that the peptides were hydrolysed outside of the cell. No growth tests using aspartyl-peptides have been reported for this organism.

The yeast Saccharomyces cerevisiae was found in early studies to be able to utilise peptides as sources of nitrogen (Harris, 1958; Suomalainen & Oura, 1971). Lysine auxotrophs could grow on lysine but not on dilysine, tri-lysine or higher oligomers of this amino acid (Becker et al., 1973). Further studies carried out using methionine, leucine and lysine auxotrophs showed that S. cerevisiae could utilise di- and oligopeptides containing all these amino acids for growth.

The data therefore shows that for all microorganisms studied so far, peptides can serve as sources of either required amino acids or nitrogen. This capacity for peptide utilisation is indicative of the central position of the constituent amino acids in cellular metabolism in both bacteria and fungi, as precursors involved in protein biosynthesis and as sources of other metabolites.

1.4 Uptake and Transport of Peptides by Microorganisms

Peptide utilisation could theoretically occur via one of two fundamentally different pathways. The peptides could be hydrolysed outside the bacterial or fungal cell by extracellular or periplasmic peptidases, and the free amino acids would then be transported into the bacteria by amino acid permeases. In such a system no peptide uptake would be necessary. Alternatively, the peptides themselves could enter the cell and peptidase cleavage would then occur intracellularly. Although some extracellular peptidases have been identified in certain organisms, for all microbial systems in which detailed studies have been carried out, the evidence supports the second pathway of utilisation (Sussman & Gilvarg, 1971; Payne & Gilvarg, 1978; Payne, 1980). Such detailed studies have been undertaken in both bacterial and fungal systems. As with studies on fungal peptide utilisation, most studies of peptide transport in these organisms have concentrated on *N. crassa* and *S. cerevisiae*. Peptide uptake and transport has been thoroughly examined in Gram-negative bacteria, in particular *E. coli* whereas few studies have been reported in this area for Gram-positive systems. This section looks in detail at peptide uptake and transport systems required if exogenously supplied peptides are to be hydrolysed by peptidase enzymes, in particular in the Gram-negative bacteria *E. coli* and *S. typhimurium*, but also in fungi.

The Gram-negative bacteria *E. coli* and *S. typhimurium*

comprise at their outermost surface a distinct membrane, the outer membrane underlying this is a peptidoglycan layer and between this and the cytoplasmic membrane (inner membrane) lies the periplasmic space. The outer membrane and the peptidoglycan together constitute the cell wall. Some time ago the cell wall of *E. coli* was shown to have a molecular sieving property that dramatically affected peptide uptake. The ability of various amino acid auxotrophs to grow on a series of homologous oligopeptides ceased abruptly at a particular point in the series (Payne & Gilvarg, 1968). The nutritional cut off did not always occur at the same homologue, and therefore sieving was not directly dependent on a particular number of amino acid residues in the peptide chain. Measurement of peptide size, using Sephadex Gel Filtration, indicated that the overall volume (stokes radius) was the feature that determined whether or not a peptide substrate could be utilised, the cut off coming at about the size of the model substrate pentalysine (M_r = 650d). Other workers have since presented evidence for a size limit on bacterial peptide uptake by *E. coli* (Smith *et al.*, 1970), *Strep. lactis* (Rice *et al.*, 1978) and *P. putida* (Cascieri & Mallette, 1976a). It is now clear that the sieving function is carried out in *E. coli* by the outer membrane porins *Omp*F and *Omp*C which have been found to be required for the movement of peptides across the outer membrane and which exhibit some limited substrate specificity (Andrews & Short, 1985). Elimination of these proteins from the *E. coli* outer membrane results in a cell in which the primary means for peptide permeation have been lost.

Early studies on bacterial nutrition indicated that amino acids and peptides had different routes of uptake, and this distribution was further emphasised by the isolation of E. coli mutants that failed to transport a particular amino acid but responded normally to the same amino acid in dipeptide form (Payne & Gilvarg, 1971; Payne, 1976). Mutants of E. coli were also isolated which failed to utilise oligopeptides but responded normally to amino acids and dipeptides (Payne, 1968). Studies on mutants deficient in oligopeptide and dipeptide uptake led to the description of a further tripeptide transporting system (Barak & Gilvarg, 1975). Proteins involved in peptide uptake have been localised to the cytoplasmic membrane and it has been clearly demonstrated that it is transport across this membrane which is the rate limiting step in peptide utilisation (Payne & Bell, 1979).

The 3 distinct peptide transport systems have been defined both in E. coli and S. typhimurium. They have different but overlapping substrate specificities (Higgins, 1984; Higgins & Gibson, 1986; Manson et al., 1986). The oligopeptide permease (Opp) is the major peptide transport system under aerobic conditions. It handles any peptide of up to 5 amino acid residues with little specificity for the amino acid composition of the peptide (Payne & Gilvarg, 1968; Payne & Bell, 1979). The tripeptide permease (Tpp) was originally defined as a minor system with restricted specificity for hydrophobic peptides. However it is now clear that Tpp will transport most tripeptides

as well as certain dipeptides albeit with greater affinity for peptides containing hydrophobic amino acids (Gibson et al., 1984; Higgins & Gibson, 1986). Tpp is transcriptionally activated under anaerobic conditions or by exogenous leucine to become a major transport system (Jamieson & Higgins, 1984, 1986). The third peptide transport system, the dipeptide permease Dpp has a marked preference for dipeptides. Dpp is a binding, protein dependent transport system and in addition to its role in transport, the binding protein serves as a chemoreceptor for chemotaxis (Manson et al., 1986).

Four genes required for the oligopeptide permease have been mapped in a single operon at 35 minutes on the S. typhimurium chromosome and at 27 minutes in E. coli (Higgins et al., 1983; Hogarth & Higgins, 1983). The first gene of the operon oppA encodes a periplasmic protein of molecular weight 58,800 (Higgins & Hardie, 1983; Guyer et al., 1985; Hiles & Higgins, 1986). This is one of the most abundant proteins in the periplasm. The three promoter genes, oppB, oppC and oppD encode membrane associated proteins (Hiles et al., 1987). In addition to its nutritional role Opp serves an important function in the recycling of cell wall peptides released from peptidoglycan during growth (Goodell & Higgins, 1987). The oligopeptide transport system also provides a route for the uptake of many peptide antibiotics, both natural and synthetic (Ringrose, 1980). The whole operon encoding the 4 oligopeptide permease genes from S. typhimurium has recently been cloned and characterised (Hiles

et al., 1987) and this should facilitate further regulatory physiological and biochemical studies of peptide uptake.

No peptide is known which is specifically transported by the dipeptide permease, but dpp mutations have been isolated in an Opp$^-$ background using the peptide antibiotic bacilysin produced by certain Bacillus strains. These mutations in dpp map at 82 min on the S. typhimurium chromosome and the mutant strains are defective in the uptake of a wide variety of dipeptides as well as some tripeptides (Higgins & Gibson, 1986).

Early studies showed that derivatisation of the N-terminal α-amino group impaired the utilisation of peptides in E. coli and this was subsequently shown to be a function of transport system requirement for the unsubstituted α-amino group (Gilvarg & Katchalski, 1965). In later studies it was shown that E. coli utilises mono-α-N-substituted peptides in which the positive charge is retained. This specificity applies to both dipeptides and oligopeptides. Similar conclusions have been reached with various strains of E. coli (Becker & Naider, 1974), Pseudomonas (Cascieri & Mallette, 1976a; Miller & Becker, 1978), and S. typhimurium (Jackson et al., 1976).

The E. coli peptide permeases do not have an absolute requirement for the C-terminal α-carboxyl group (Payne & Gilvarg, 1968; Payne, 1973) and will take up peptides with this group variously derivatised. However the modifications are not

without effect as the derivatives are less well transported than the parent molecules. The specificity of the dipeptide permease appears to be more strict than with the oligopeptide permease. Thus uptake of dipeptides with this group absent or derivatised seems to be via the oligopeptide system as it is competitively affected by oligopeptides and does not occur in mutants lacking the oligopeptide system.

Growth tests have indicated that the peptide transport systems show specificity for L-residues, and that α-peptide bond linkages are required for peptide uptake in E. coli K12. Evidence from diverse studies indicate that transport systems are able to handle peptides carrying all types of amino acid side chains (Barak & Gilvarg, 1976; Payne, 1975; Payne & Gilvarg, 1971, 1978) although the kinetic effect of different structures on transport remains unclear. With both dipeptides and oligopeptides competitive inhibitions of auxotrophic growth have been reported for many species including P. maltophilia (Cascieri & Mallette, 1976a) and E. coli K12 (Payne, 1968).

Few studies have been carried out on the regulation of peptide transport in bacteria. Amino acid permeases are in almost all cases constitutively expressed although inducible amino acid permeases for tryptophan in E. coli and proline in S. typhimurium have been identified. The rate of peptide uptake was shown to be neither increased nor decreased when peptide concentration in growth media was varied, and the oligopeptide

uptake system of S. typhimurium has been found to be consitutively expressed (Jamieson & Higgins, 1984; Hiles et al., 1987). As mentioned above the tripeptide permease has been found to be activated by both anaerobic conditions or the presence of exogenous leucine in growth media.

Levels of nitrogen and carbon are likely to play a role in the regulation of peptide transport. Limiting levels of nitrogen have been shown to lead to enhanced levels of amino acid binding proteins and glutamine synthase, and a similar effect may arise for peptide binding proteins under similar conditions. The effect of nitrogen and carbon limitation on bacterial peptide transport are poorly understood and much work remains to be done if their effects and mechanism of regulation are to be elucidated.

Information on peptide uptake in Gram-positive organisms is sparse. The cell envelopes of Gram-positive organisms lack an outer membrane, have a thicker peptidoglycan layer than that found in Gram-negative organisms, contain teichoic acid, and lack a periplasmic space although an apparently analogous membrane wall interlayer has been described (Giesbrecht et al., 1977). The penetrability of cell walls from B. megaterium to compounds of graded size has been determined (Gerhardt & Judge, 1964; Scherrer & Gerhardt, 1964). Dependent on the size of molecule, uptake could be observed in 3 distinct regions. The interstitial volume between the cell walls was the only compartment to which

compounds of molecular mass greater than 30,000 could gain access. Compounds in the molecular mass range 1,500-30,000 could enter an additional space, taken to represent the cell wall while only the smallest molecules could penetrate into the cell cytoplasm. The results were taken as an indication of molecular sieving by the cell membranes, which would allow penetration to the cell wall of small peptides. Dipeptide and tri-peptide uptake have been examined in the Gram-positive lactobacilli (Guirard & Snell,.1962) but the molecular basis of this uptake remains poorly understood.

Molecular sieving studies with the cell walls of the fungus N. crassa suggested that unlike the Gram-negative bacterial cells this structure in fungi might not restrict access by peptides to a membrane localised transport mechanism (Trevithick & Metzenberg, 1966a, b). If this were correct, then the inability of amino acid auxotrophs of Neurospora to grow on large e.g. penta- or hexa- peptides might be taken as evidence for an upper size limit for transport by a peptide transport system. In order to find out whether it was the cell wall that restricted the access of larger peptides, a mutation (os-1) was crossed into a leucine auxotroph of N. crassa and the resulting double mutant, used in growth assays on peptides (Wolfinbarger & Marzluf, 1975). The os-1 mutation results in larger pores in the cell wall, such that molecules approaching a molecular weight of 18,500d can permeate the cell wall, as opposed to 4,750d for the wild type. The double mutant was unable to grow on peptides larger than a

pentapeptide and it was concluded that unlike in bacterial cells, Neurospora and perhaps other fungal cells are restricted in growth on larger peptides by an inability of the transport mechanism itself to accommodate peptides exceeding a hydrodynamic volume similar to that of tri-leucine. Such a hydrodynamic volume would apply to a pentapeptide of mixed amino acid residues.

Evidence in support of the presence of a single oligopeptide transport system in Neurospora came from studies of a mutant strain unable to grow on any leucine containing peptide as a source of leucine. Growth occurred only on those fractions containing leucine (Wolfinbarger & Marzluf, 1975). This presumed removal of the oligopeptide transport system in Neurospora resulted in an inability to grow on peptides ranging from pentapeptides to tripeptides. As dipeptides are not taken up well, if at all by N. crassa, it was concluded that in N. crassa there was probably one oligopeptide transport mechanism at least for leucine containing peptides.

In S. cerevisiae evidence presented so far, suggests that as in Neurospora, there is a single peptide transport system. Dipeptides and tripeptides have been shown to undergo competition for transport. For example the utilisation of the leucine dipeptide Leu-Leu by S. cerevisiae was greatly inhibited by the dipeptide and the tripeptide Met-Met or Met-Met-Met (Marder et al., 1977). In addition, it proved possible using a one step

selection procedure to isolate a mutant of S. cerevisiae defective in the ability to take up both a dipeptide and tripeptide (Nisbet & Payne, 1979a).

The S. cerevisiae peptide transport system has highest affinity for peptides containing hydrophobic amino acids and is capable of taking up peptides composed of a variety of amino acids. Unlike E. coli, growth response appears to be sensitive to both the composition and sequence of the peptide substrate however it has not been defined whether this effect is mediated through the transport process or through intracellular hydrolysis.

Several studies have established that in S. cerevisiae peptides are not hydrolysed prior to their active transport into the cell (Naider et al., 1975; Marder et al., 1977; Parker et al., 1980). The accumulation of intact non-hydrolysable subtrates has been demonstrated in S. cerevisiae (Nisbet & Payne, 1979b). The transport of several radioactive di- and tri-peptides in S. cerevisiae has been studied (Moneton et al., 1986). A peptide transport-deficient mutant isolated on the basis of its resistance to nikomycin, a toxic non-hydrolysable peptide, lost most of its capacity to take up di- and tri-peptides. Analysis of transport kinetics showed that peptide transport was not dependent on intracellular hydrolysis. The ability of S. cerevisiae to transport and utilise peptides appeared to be a non-inducible characteristic.

In response to peptides, added to the growth medium, no evidence was found for induced synthesis of peptidases or peptide transport systems in strains examined. On the other hand growth in the presence of ammonium ions appears to repress the transport of peptides in S. cerevisiae (Becker & Naider, 1977). Cells grown in medium with ammonium nitrate as nitrogen source take up radiolabelled methionine at a reduced rate compared to cells grown on proline or glutamic acid as nitrogen source. This phenomenon of nitrogen catabolite repression has also been shown for general amino acid permeases in yeast.

In contrast to the position in S. cerevisiae, the pathogenic fungus Candida albicans has been shown to have multiple peptide transport systems and multiple permeases (Sarthou et al., 1983; Yadan et al., 1984). A C. albicans mutant resistant to nikomycin, which had lost dipeptide transport was found to have an increased capacity to take up tripeptides. Other studies produced conflicting data on the multiplicity of C. albicans peptide permeases (Logan et al., 1979; Davies, 1980) but studies using novel chromophoric peptides have confirmed the presence of at least 2 peptide transport systems, one for dipeptides, and the other for oligopeptides (McCarthy et al., 1985).

1.5 Microbial Peptidase Enzymes: Their Structure, Biochemistry, Genetics & Regulation

The evidence for the uptake and utilisation of peptide substrates in microbial systems has been reviewed in the previous sections. The subsequent hydrolysis of these peptides is mediated by peptidase enzymes. The early work on the growth response of bacteria, in particular that of *E. coli* amino acid auxotrophs to small peptides, led to the realisation that peptides must be hydrolysed before their constituent amino acids became available to the cell (Fruton & Simmonds, 1950). Subsequent work in several laboratories showed that peptides containing almost any of the standard L-amino acids or glycine could be hydrolysed by whole cells, cell extracts and partially purified preparations from *E. coli* K12 (Meisler & Simmonds, 1963; Simmonds, 1966, 1970, 1972; Sussman & Gilvarg, 1969) and various peptidase enzymes have since been identified as responsible for these hydrolyses.

Although a small number of fungal peptidases have been identified and partially characterised (Ichishima *et al.*, 1983; Achstetter *et al.*, 1985) few detailed investigations have been carried out on these fungal enzymes. This section therefore looks at the properties of the peptidase enzymes identified in bacterial systems with particular emphasis on the peptidases of the well studied Gram-negative strains *E. coli* K12 and *S. typhimurium*. Of these peptidases, those capable of hydrolysing

aspartyl-peptides are of particular interest in this project and will be given special emphasis. A number of studies have localised E. coli K12 peptidase activity towards different substrates in the cell cytoplasm (Van Lenten & Simmonds, 1967; Simmonds & Toye, 1967) as well as individual peptidase enzymes (Ladzunski et al., 1975; Murgier et al., 1976) and so the E. coli K12 and S. typhimurium peptidases discussed in this section are all considered to be intracellular peptidases.

E. coli K12 and S. typhimurium produce at least 10 different peptidase enzymes (Miller, 1987) including at least 2 enzymes that appear to be C-terminal exopeptidases (dipeptidyl carboxypeptidase and oligopeptidase A), a broad specificity dipeptidase, peptidase D, a broad specificity tri-peptidase (peptidase T), 3 broad specificity N-terminal exopeptidases (peptidases N,A,B), as well as 2 peptidases specific for the degradation of prolyl peptides (peptidases P and Q). In addition, a glycyl-glycine dipeptide hydrolysing enzyme (peptidase G), an aspartyl-specific peptidase (peptidase E) and a peptidase specific for the removal of methionine residues from polypeptide chains (peptidase M) have been identified as yet only in S. typhimurium. A β-aspartyl peptidase has also been identified as yet only in E. coli (Haley, 1968), as well as a poorly characterised aminopeptidase (Simmonds et al., 1976). D-amino acid carboxypeptidases and peptidases have been identified in both Gram-positive and Gram-negative organisms, and have specificity towards the cell wall peptides containing

D-amino acid residues (Blomberg & Strominger, 1974).

Biochemical studies of these E. coli K12 and S. typhimurium peptidases have revealed a diverse range of structures, substrate specificity and other properties although the broad substrate range peptidases and the dipeptidases show a large degree of overlapping specificity (Miller & Schwartz, 1978; Kirsch et al., 1978). The broad specificity dipeptidase, peptidase D, was identified in both organisms as a band of peptidase activity on polyacrylamide gels (Relative mobility, 0.55) with activity towards the dipeptide Leu-Gly but with no activity towards the tri-peptide Leu-Gly-Gly (Miller & Mackinnon, 1974; Miller & Schwartz, 1978). Isolations and purification of peptidase D have been carried out in both organisms and the enzyme's activity has been found to be dependent on the presence of Mn^{2+} or Co^{2+} ions and β-mercaptoethanol, and inhibited by Zn^{2+} ions and the metal-ion chelating agent EDTA (Simmonds et al., 1976; Kirsch et al., 1978; Hermsdorf, 1978). The molecular mass of the S. typhimurium peptidase D has been determined at 158 kd (Kirsch et al., 1978) whereas the molecular weight of the dimeric E. coli K12 enzyme has been determined at 100 kd with a sub-unit molecular weight of 52 kd (Klein et al., 1986). Not all dipeptides were found to be hydrolysed by the peptidase D enzyme; highest activity was observed with the substrate Met-Ala (or Met) and low activity with Leu-X and Lys-X dipeptides.

The broad specificity tripeptidase, peptidase T, (also

referred to as aminotripeptidase) was first discovered during a study of trilysine metabolism in E. coli (Sussman & Gilvarg, 1970). Characterisation of this enzyme showed that it was capable of cleaving an amino terminal leucine, lysine, methionine or phenylalanine residue from certain tripeptides and that activity towards dipeptides, peptide amides, substituted peptide esters and tetrapeptides could not be demonstrated from purified peptidase T (Hermsdorf, 1978). The molecular mass of the E. coli K12 enzyme was estimated at 80 kd and activity was found to be dependent on Mn^{2+} and inhibited by Zn^{2+}. The E. coli K12 peptidase T activity was found to correspond with a band of tripeptidase activity observed in polyacrylamide gels (mobility 0.4), of extracts from S. typhimurium (Miller & Mackinnon, 1974).

The broad specificity, peptidase A (also known as aminopeptidase I) degrades di-, and tri-peptides with free N-termini, and the E. coli K12 enzyme has been purified 2,000 fold (Vogt, 1970). This enzyme has a molecular mass of 323 kd with a sub-unit molecular mass of 52 kd. It is inhibited in activity by Zn^{2+} and inactivated by dialysis against EDTA, though after EDTA treatment activity can be fully restored by addition of either Mn^{2+} or Mg^{2+}. The E. coli K12 peptidase A is stable to incubation at high temperature (3 minutes at 75°C). Peptidase A type enzymes have been identified in both E. coli B (Matheson & Dick, 1970; Matheson et al., 1970; Dick et al., 1970) and S. typhimurium (Miller & Mackinnon, 1974). The E. coli K12 and S. typhimurium enzymes have similar mobilities on polyacrylamide gels.

The peptidase denoted peptidase B (also known as aminopeptidase AP) was initially identified due to its Met/Leu aminopeptidase activity in E. coli K12 extracts (Sussman & Gilvarg, 1970). It was localised in polyacrylamide gel activity strains as a band (mobility 0.3) showing activity towards both Leu-Gly-Gly and Leu-Gly in both E. coli K12 (Miller & Schwartz, 1978) and S. typhimurium (Miller & Mackinnon, 1974). Both activities were found to be Mn^{2+} dependent and inhibited by Co^{2+}, Zn^{2+} ions as well as EDTA. Its activity is also reported to be stimulated by β-mercaptoethanol. Peptidase A appears to have a broad substrate specificity, hydrolysing both dipeptides and tri-peptides, but the former more slowly than the latter (Hermsdorf & Simmonds, 1980). The sub-unit molecular mass of the E. coli K12 enzyme has been determined at 230 kd (Hermsdorf & Simmonds, 1980), and the holoenzyme molecular mass at 230 kd (Hermsdorf, 1978) suggesting that this enzyme is a monomer.

A Leu-Leu dipeptide hydrolysing enzyme, designated aminopeptidase L has also been identified in E. coli K12 (Simmonds et al., 1976). This enzyme has a reported molecular mass of 230 kd and has been identified in polyacrylamide gel stains as a variable band of activity associated with peptidase B, at mobility 0.28, with specificity for both Leu-Gly and Leu-Gly-Gly (Miller & Schwartz, 1978).

A further peptidase enzyme designated peptidase N with broad specificity but alone in its ability to degrade pseudo

peptide chromogenic substrates has been identified in E. coli K12 (Yang & Somerville, 1976; Miller & Schwartz, 1978) and S. typhimurium (Miller & Mackinnon, 1974). This peptidase appeared as an activity band (mobility 0.6) on polyacrylamide gels. Separate studies have produced differing molecular mass values for the E. coli K12 peptidase N at 100 kd (Yang & Somerville, 1975) and 89 kd (McCaman & Villarejo, 1982). A calculated molecular mass of 99 kd has been proposed based on nucleotide sequence data (Foglino et al., 1986). The earlier studies both concluded that the peptidase is monomeric, that it exhibits a broad pH range of activity and is inhibited in its activity by Zn^{2+}, Fe^{3+}, Cr^{3+} ions and EDTA. Conflicting conclusions were also drawn on the effect of sulphydryl reagents on enzyme activity, with one group reporting inhibition and the other claiming no effect. Both studies showed no peptidase N inhibition by serine protease inhibitors and, in contrast to peptidase A, activity was rapidly lost on incubation at 70°C. The E. coli K12 enzyme degraded both dipeptides and tripeptides but has higher dipeptidase activity.

The aspartyl-specific peptidase E from S. typhimurium was identified as a band of activity (mobility 0.7) in polyacrylamide gels (Carter & Miller, 1984). It was found to degrade all N-terminal L-α-aspartyl peptides except for Asp-Pro, but was inactive towards dipeptides with N-terminal asparagine or glutamate, and C-terminal aspartyl peptides like Phe-Asp. The activity of this monomeric 35 kd enzyme appeared to be unaffected

by metal ions or metal ion chelating agents as shown by qualitative tests. A β-aspartyl peptidase has been identified in cell extracts from E. coli (Haley, 1968). Like the S. typhimurium peptidase E, this peptidase did not require metal ions for its activity nor was its activity effected by sulphydryl inhibitors or metal ion chelating agents. This 120 kd enzyme was specific for α-aspartyl dipeptides only.

An N-terminal methionine-specific aminopeptidase has only recently been identified in S. typhimurium (Miller et al., 1987). The activity of this 34 kd peptidase is stimulated by Co^{2+} and is inhibited by EDTA. Unlike other Salmonella and E. coli K12 peptidases, this enzyme activity could not be detected by activity staining after electrophoresis of crude extracts on non-denaturing gels. The methionine specific peptidase (designated peptidase M) was found to hydrolyse methionine residues from both small peptides and a larger monomeric protein, unprocessed interleukin 1.

Other peptidases from E. coli K12 and S. typhimurium are less well characterised. Peptidase P (called also aminopeptidase P) has been identified in both organisms (Miller & Mackinnon, 1974; Miller & Schwartz, 1978) and hydrolyses only peptide bonds where the nitrogen atom is contributed by a proline residue (X-Pro peptides). The peptidase P enzyme from E. coli K12 has been purified and found to have a molecular mass of 230 kd (Yaron & Berger, 1970). An aminopeptidase, designated peptidase Q, has

also been isolated in these organisms and catalyses removal of N-terminal proline from both large and small peptides (McHugh & Miller, 1974; Miller & Schwartz, 1978; Miller & Mackinnon, 1974). Dipeptidyl carboxypeptidase enzymes have been identified in E. coli K12 and S. typhimurium and these enzymes found to cleave dipeptides from the C-terminal ends of peptides (Yaron et al., 1972; Vimr & Miller, 1983). N-blocked tripeptides are hydrolysed to yield an N-blocked amino acid and a dipeptide. Tetrapeptides are cleaved to yield 2 dipeptides. A free carboxy-terminus is required for hydrolysis. Finally, an oligopeptidase that hydrolyses N-acetyl-$(Ala)_4$ has been identified in cell extracts of S. typhimurium (Vimr et al., 1983). This oligopeptidase A has specificity towards certain N-blocked tetrapeptides, unblocked pentapeptides and unblocked hexapeptides usually but not always liberating the C-terminal tripeptide.

The broad specificity peptidases N, A, B and D have been implicated in the degradation of Asp-Leu and other L-α-aspartyl peptides in E. coli K12 (Miller & Schwartz, 1978). Analysis of peptide utilisation profiles for amino acid auxotrophic strains of E. coli K12 showed that wild type strains could utilise the dipeptide Asp-Leu as sole source of required leucine, whereas a mutant strain lacking all of the peptidases N, A, B, D and Q could not grow on this substrate. Genetic techniques were used to introduce single peptidase alleles into the peptidase deficient mutant. Strains carrying a single one of the

peptidases N, A, B and D were reported to gain the ability to utilise Asp-Leu as a source of leucine.

Contrary to these findings it was discovered that in S. typhimurium the only dipeptide serving as a histidine source for a strain lacking peptidases N, A, B and D was Asp-His (Kirsch et al., 1978), and the only leucine containing peptide found to serve as a leucine source for a mutant leucine auxotroph lacking peptidases N, A, B, D, P and Q was Asp-Leu (Carter & Miller, 1984). These observations led to the identification of a peptidase with narrow specificity for L-α-aspartyl peptides, peptidase E, in S. typhimurium. The only other S. typhimurium peptidase found to hydrolyse L-α-aspartyl peptides was the enzyme designated peptidase B.

The genes encoding all of the broad specificity peptidases and the aspartate-specific peptidase E have been mapped on the chromosome of S. typhimurium (Sanderson, 1988). In E. coli K12 all of the broad specificity peptidases except peptidases A and B have been mapped (Bachmann, 1983). The genes encoding the different peptidases were located all around the genome. Peptidase D mutants have been isolated in both organisms, in E. coli K12 as strains deficient in peptidases A and N which become resistant spontaneously to the dipeptide Val-Gly, (Miller & Schwartz, 1978), and in S. typhimurium as strains deficient in peptidases A and N which after penicillin selection lost the ability to utilise the dipeptide Leu-Gly as a source of the amino

acid leucine (Miller & Mackinnon, 1974). In both organisms the pepD mutations mapped at position 6 minutes on the genome near the ProAB locus (Miller 1975a, Miller, 1975b). The gene encoding the E. coli K12 peptidase D has been cloned in the plasmid vector pUC18 utilising an unusual peptidase D property, the ability to hydrolyse the abnormal dipeptide carnosine (β-alanylhistidine) for selection of clones (Klein et al., 1986). The gene has not yet been sequenced.

Mutations in the gene encoding the S. typhimurium peptidase A were selected as peptidase N deficient leucine auxotrophs which failed to utilise Leu-Ala-NH_2 as a leucine source. The pepA marker was found to be co-transduced with the markers pyrB and argI located at 0 minutes on the genome (Miller, 1975a). S. typhimurium peptidase B mutations were isolated in pepN$^-$pepA$^-$ pepD$^-$ backgrounds as strains deficient in the ability to use the dipeptide Leu-Leu as a leucine source (Miller & Mackinnon 1974). Transposon technology was used to map the peptidase B locus between the genes strB and GlyA on the S. typhimurium genome, at position 56 minutes (Green & Miller, 1980), but now adjusted with other markers in this region of the S. typhimurium genome to map position 53 minutes (Sanderson, 1988). Isolation of pseudorevertants from an S. typhimurium strain carrying stable mutations in pepN, pepA and pepB, which were able to use Leu-Leu-Leu as a leucine source led to the mapping of the gene encoding the Leu-Leu-Leu cleaving enzyme peptidase T (Strauch et al., 1983). The pepT gene mapped at position 25 minutes on the genome.

Mutant strains deficient in peptidase N have been isolated as strains unable to degrade L-alanyl-β-napthylamide (Miller & Mackinnon, 1974; Miller & Schwartz, 1978). The gene encoding peptidase N has been mapped precisely in E. coli K12 at position 20.8 minutes on the genome (Latil et al., 1976; Feutrier et al., 1982; McCaman et al., 1982; Bally et al., 1984a), and at approximately 20 minutes on the S. typhimurium genome (Miller, 1975a; Bachmann, 1983). The E. coli K12 pepN gene has been cloned on a multicopy plasmid vector (Bally et al., 1983; Bally et al., 1984a) and the exact physical location of the gene determined as well as the direction of pepN transcription (Bally et al., 1984b). Sub-cloning of DNA restriction fragments carrying the pepN gene has led to the nucleotide sequencing of the gene (Foglino et al., 1986).

Genetic mapping placed the pepE locus at 91.5 minutes on the S. typhimurium genetic map and established the gene order metA pepE malB. Mutations in the pepE gene were isolated as strains with reduced levels of Asp-Leu degrading activity as determined using a peptidase microassay (Carter & Miller, 1984).

Detailed studies on the regulation of synthesis, expression and activity of individual peptidase enzymes have only been carried out with E. coli K12 peptidase N and the S. typhimurium tripeptidase T. Early studies on peptidase regulation were hampered by the presence of numerous peptidases with overlapping substrate specificities although it soon became obvious that the

intracellular peptidases were constitutive enzymes whose synthesis and activity *in vivo* could be subject to several types of regulation and varied with the phase of the bacterial cell cycle. Evidence for the constitutive nature of *E. coli* peptidase synthesis came for the most part from assays for peptidase activity in crude extracts made from cells cultured in peptide-free media. Where tested for comparison, growth on peptide containing media had no significant effect on the level of peptidase activity in the resultant cell extracts (Payne, 1972a, b; Sarid, *et al.*, 1962; Simmonds, 1970, 1972; Sussmann & Gilvarg, 1970; Vogt, 1970). Peptidase activity was found however to vary in response to environmental conditions. Extensive studies with *E. coli* involving chemostat-grown cultures and activity tests carried out in several assay systems have shown clearly that demonstrable dipeptidase activity varies in response to environmental changes such as carbon-source limitation, which produced cells with low activity (towards the dipeptides Gly-Gly, Pro-Gly, and Val-Val), and oxygen depletion which produced cells with high activity (Payne, 1972b). While ammonium ion-limited media resulted in cells showing an intermediate level of dipeptidase activity, these cells contained the least activity towards tri-glycine. In addition it was observed that phosphate limitation produced higher levels of both dipeptidase and tripeptidase activity (Payne 1972b).

In chemostat grown cultures, peptidase activities of *E. coli* strain B cell extracts were independent of the pH of the

growth medium in the range pH 5.5-7.0 (Payne, 1972b). Different results were obtained with batch grown cultures of strain E. coli K12 where cell extract dipeptidase activity (towards Gly-Leu, Leu-Gly and Phe-Gly) decreased after the cessation of culture growth and the pH of the culture fell below pH 5.5 (Simmonds, 1970). The same result was evident after exponential phase cultures were acidified to pH5 by the addition of HCl but only if the growth medium was deficient in 'trace metals'. Exposure of cells grown in a metal-deficient culture medium to relatively high concentrations of Zn^{2+} likewise caused a fall in the dipeptidase activity of cell extracts. These effects were attributed to the inactivation of peptidases that were present in the cells before acid or Zn^{2+} treatment. As many E. coli K12 peptidases appear to be metalloenzymes the activity of which in vitro is inhibited by Zn^{2+} this suggests that divalent cations may play a role in the regulation of enzymic activity in vivo (Payne, 1972a, b).

Replacement of glycerol by glucose in a peptone-containing medium has also been found to alter the apparent dipeptidase activity of strain E. coli K12 (to Leu-Gly and Phe-Gly). Peptidase activity in the ribosomal fraction from glucose-grown cells showed less Leu-Gly hydrolysing activity than was seen with glycerol grown cells. Furthermore only in glucose grown cells did the activity of each subcellular fraction alter after the cessation of exponential culture growth. These changes may be due to the acid-inactivation effect since cultures growing in

glucose showed a significant decrease in pH at the end of the exponential growth phase (Simmonds, 1970).

Studies on the regulation of peptidase synthesis, looking specifically at levels of E. coli K12 peptidase N activity have shown that this enzyme is synthesised constitutively (McCaman et al., 1982; Ladzunski et al., 1975b). Starvation of E. coli K12 cells for carbon, nitrogen or phosphate were reported to have no effect on peptidase N activity levels (McAman et al., 1982). Other studies have shown however that under conditions of phosphate limitation K12 peptidase N levels may be altered (Ladzunski et al., 1975a; Gharbi et al., 1985). Fusion of the pepN promoter to the E. coli K12 chromosomal malQ gene on a multicopy plasmid gave 5-fold to 8-fold increases in amylomaltose production under conditions of phosphate limitation (Foglino & Ladzunski, 1987). Oxygen limitation was also found to increase peptidase N activity levels in E. coli K12 (Gharbi et al., 1985; Foglino & Ladzunski, 1987). Contradictory data and conclusions have also been presented on the effect of different carbon sources and media on levels of peptidase N activity with one report concluding there was no alteration in enzyme activity (McCaman et al., 1982) and others reporting variation in activity levels dependent on carbon sources supplied (Murgier & Gharbi, 1982; Gharbi et al., 1985). Similarly some researchers report no alterations in peptidase N activity during the E. coli K12 growth cycle (McAman et al., 1982) while other researchers have reported an increase in activity levels in stationary phase cells (Yang & Somerville, 1975).

The effect of oxygen limitation (anaerobiosis) on peptidase T levels in S. typhimurium has been examined, and this activity was found to be induced under these conditions (Strauch et al., 1985). Peptidase T was synthesised constitutively but was found to be present at higher levels in late exponential and stationary phase cells. Starvation of S. typhimurium cells for carbon, nitrogen or the amino acid leucine did not effect the level of peptidase T activity. Mutations in 2 loci oxrA and oxrB were found to prevent induction of the pepT gene. A recent study has shown that peptidase T activity is raised in cells exposed to osmotic stress (Ni Bhriain et al., 1989).

Numerous peptidase enzymes have been identified in Gram-positive bacteria and those for which preliminary characterisations have been carried out, like the peptidases of E. coli K12 and S. typhimurium appear to be metallopeptidases of varying structure and specificity, examples being the 3 aminopeptidases of Bacillus stearothermophilus (Roncari, et al., 1975). Extracellular peptidases which are also metallopeptidases, have been identified in Bacillus licheniformis (Hall et al., 1966), Bacillus subtilis (Ray & Wagner, 1972) and Aeromonas proteolytica (Litchfield & Prescott, 1970). The uptake of peptides in Gram-positive bacteria is poorly understood and studied and it is not known whether the presence of these extracellular peptidases is combined with an absence of peptide uptake systems. In the fungal strain N. crassa extracellular peptidase activities have been identified side by side with peptide uptake systems (Wolfinbarger & Marzluf, 1974).

The peptidases of E. coli K12 and S. typhimurium appear in general to be metalloenzymes, a feature shared with intracellular and extracellular peptidases from a number of other bacteria. The E. coli K12 and S. typhimurium enzymes have varying and often overlapping specificities with some peptidases possessing a very narrow substrate range and others capable of hydrolysing a broad range of peptide substrates. There are no noticeable similarities in the physical structures of these peptidases. Those for which regulatory studies have been performed appear to be synthesised constitutively, and activity may be stimulated by oxygen limitation. Regulatory data in many cases is contradictory.

The particular biochemical, regulatory and genetic characteristics of peptidases are most likely linked to the role of these enzymes in microbial cells. The following section considers the functions of the peptidases in microbial cells.

1.6 The Functions of Microbial Peptidases

The cellular roles of bacterial peptidases have been discussed in a number of reviews (Miller, 1975b; Hermsdorf & Simmonds, 1980; Miller, 1987). The roles of fungal peptidases are less well studied but examination of the data available reveals that bacterial and fungal peptidases share a number of common functions, being involved in the utilisation of exogenous peptides for nutritional purposes, the turnover of intracellular proteins, the degradation of abnormal proteins or toxic peptides and the processing of nascent polypeptide chains.

Following the early demonstrations of peptide utilisation by *E. coli*, the presence of peptide hydrolytic activity in whole cells and cell extracts, and the identification of specific peptidases in this microorganism, final confirmation of the role of peptidases in the utilisation of exogenously supplied peptides came through the isolation of mutant strains of both *E. coli* K12 (Miller & Schwartz, 1978) and *S. typhimurium* (Miller & Mackinnon, 1974) lacking peptidase enzymes. These mutant strains were found to lose the capacity to utilise particular peptides as sources of amino acid growth requirements. Peptidase enzymes have been similarly implicated in the nutritional degradation of peptides in Gram-positive *Lactobacilleae* (Payne, 1976).

Further studies have shown that bacterial peptidases provide protection against deleterious effects of uncleaved

peptides which may accumulate intracellularly and inhibit bacterial growth. For example bacteriostatic leucyl peptides extend the length of lag phase prior to the onset of exponential phase in E. coli K12 but have greater inhibitory effect on aged cells, which have decreasing peptidase activity and hence reduced ability to cleave these peptides (Meisler & Simmonds, 1963). The tripeptide tri-lysine can normally be used nutritionally by wild type E. coli K12 but a mutation which eliminates a peptidase capable of cleaving this peptide renders the mutant sensitive to inhibition by tri-lysine (Sussman & Gilvarg, 1971). Therefore at least some uncleaved peptides can inhibit growth and peptidases provide protection against these peptides.

The ability of bacteria to degrade proteins and especially aberrant, non-functional or experimentally induced incomplete proteins is well documented (Miller, 1975; Payne, 1976; Goldberg & St John, 1976). Such degradation is evident in cells in growing cultures and is often increased after cultures enter the stationary phase or when growth is artificially limited by the use of culture media deficient in carbon or nitrogen sources. While the initial steps in proteolysis are catalysed by proteinases the complete degradation of proteins to free amino acids must also require the participation of peptidases. The role of these peptidase enzymes in the degradation of abnormal proteins has been clearly demonstrated using peptidase deficient mutants of S. typhimurium (Miller & Green, 1981). Abnormal proteins produced as a result of incorporation of the arginine

analogue, L-canavanine or generated by exposure to puromycin, were found to be rapidly degraded in wild-type strains, but in peptidase deficient strains, although also degraded, the rate of production of TCA-soluble degradation products (amino acids and peptides) was much slower. Analysis of degradation products from each strain showed that in the wild-type, the main products were amino acids whilst from the mutant strain degradation produced a complex mixture of peptides and amino acids.

The role of peptidase enzymes in the intracellular protein degradation pathway of S. typhimurium has been shown in a similar manner. A mutant of S. typhimurium deficient in 4 broad specificity peptidase enzymes showed a reduction both in the rate and extent of intracellular protein degradation in vivo during starvation and produced significant amounts of low molecular weight peptides (Miller & Zipser, 1977). In contrast the more rapid degradation of intracellular protein in the wild-type Salmonella gave rise to only free amino acids. Carbon starved peptidase deficient mutants were found to produce about a thirtieth as much amino acid from protein as the wild-type and as a result it was observed that protein synthesis was reduced in the mutant strain compared to the wild-type and that longer lag phases occurred when the mutant strain was grown in a new nutritional environment (Yen et al., 1980).

The participation of restricted specificity peptidases is also required in the bacterial protein degradation pathways of

both E. coli K12 and S. typhimurium. In the absence of 2 peptidases specific for prolyl peptides, extensive protein breakdown was observed but most of the proline originally present in the protein ended up as small peptides (Miller & Green, 1983). The presence of an aspartyl peptidase, peptidase E, mutation preventing synthesis of this enzyme in S. typhimurium, was found to have little effect on the rate of protein degradation (Carter & Miller, 1984). The fate of aspartyl peptides in the mutant strain was not investigated.

Deriving from their role in the pathway of intracellular protein degradation it has been shown that peptidase enzymes in E. coli K12 and S. typhimurium play an important role in cell survival after carbon starvation. It is well established that growing cells of E. coli K12 degrade their bulk protein at an average rate of 1-2% hr^{-1} and that when cells are subjected to starvation for carbon, nitrogen or organic nutrients the rate increases to 4-5% hr^{-1} (Mandelstam, 1960). The mechanism by which this phenomenon occurs is not understood. When a E. coli K12 wild-type culture was starved for glucose, 50% of the cells lost viability in about 6 days. When a mutant lacking 4 broad specificity peptidase activities was starved in the same manner, 50% of the cells lost viability in about 2 days (Bockman et al., 1986). Similar results were obtained with S. typhimurium (Reeve et al., 1984). This rate of loss of viability could be reduced by growing wild-type E. coli K12 or S. typhimurium in the presence of peptidase deficient mutants. It has been proposed that this

effect is due to the provision of amino acids to the wild-type starving organism in the form of peptides excreted by the mutants. These amino acids enhance the ability of the wild-type organisms to synthesise protein during starvation (Bockman et al., 1986).

A further function for peptidases may lie in the rapid degradation of mistranslation products generated during protein biosynthesis (Yen et al., 1980) although no firm evidence of their role in this process has yet been presented.

The discovery that the bacterial synthesis of proteins involves the formation of nascent polypeptide chains containing amino terminal methionine residues prompted a search for the 'methionine aminopeptidase' specifically concerned with the removal of N-terminal methionine during the biosynthesis of proteins that in their final form lack that residue. E. coli ribosomes were found to have an associated methionine aminopeptidase activity (Matheson & Murayama, 1966) but this association was determined to be artefactual on further characterisation of this activity (Vogt, 1970). Many broad specificity enzymes capable of hydrolysing methionine peptides have now been isolated but only recently has an activity specifically identified as being involved in the removal of N-terminal methionine from peptides and proteins, come to light (Miller et al., 1987). This activity, designated peptidase M, observed in S. typhimurium was found to possess the ability to

remove methionine from the N-terminus of a completed protein thus confirming its likely participation in the removal of methionine residues during peptide/protein biosynthesis.

2 so called leader peptidase enzymes have been identified in a number of different bacteria (Wolfe & Wickner, 1984; Randall & Hardy, 1986). These enzymes are involved in the removal of N-terminal peptides from integral proteins of the bacterial membrane, lipoproteins and secreted proteins. These proteins are made as precursors which bear N-terminal extensions of between 15 and 30 amino acid residues termed the leader sequence (Sabatini et al., 1982). This sequence is removed from the precursors by leader peptidases during, or shortly after insertion through the membrane. Although referred to as a peptidase, this leader peptidase shows significant endopeptidase activity and is more correctly defined as a proteinase.

Cell wall peptidoglycans are hydrolysed by specific bacterial peptidase enzymes. The D-amino acid containing peptides found in peptidoglycan are substrates for specific D-amino acid carboxypeptidases and peptidases present in both Gram-negative and Gram-positive bacteria (Blomberg & Strominger, 1974). Peptidase enzymes therefore appear to have a role in cell wall degradation and recycling of components, which has led to the suggestion, in particular in E. coli that these enzymes may have an indirect role in septum formation and cell division.

Peptidase enzymes from various fungi including Neurospora crassa (Wolfinbarger & Marzluf, 1974), S. cerevisiae (Naider et al., 1973) and Aspergillus (Nakadas et al., 1973) have been shown to be involved in the utilisation of exogenous peptides. In addition roles have been demonstrated for peptidases from S. cerevisiae in the turnover of intracellular proteins (Hansen et al., 1977), the trimming of nascent polypeptide chains (Johnson & Brown, 1974).

Peptidase enzymes have therefore been ascribed a range of functions in microbial cells. A deeper understanding of microbial metabolism may lead to further roles being assigned to these enzymes. However many workers believe that the primary role of peptidases lies in cellular protein turnover and the degradation of small peptides generated in this process (Yen et al., 1980), an idea supported by the constitutive nature of the synthesis of these enzymes. The relative physiological significance of each of the peptidase functions remains to be determined.

1.7 The Enzyme Mediated Synthesis of the Aspartyl Peptide Sweetener Aspartame: Peptidases as possible Catalysts?

One of our initial reasons for focusing on peptidase enzymes was a perceived possibility of their use as catalysts in the synthesis of the dipeptide sweetener aspartame (L-α-aspartyl-L-phenylalanine methyl ester). This sweetener has 150-200 times the sweetness of common sucrose and was discovered by chance during attempts to synthesise part of the peptide hormone gastrin (Mazur, 1969). It now lies at the centre of a multi-million pound sweetener market (Klausner, 1985). The dipeptide is a potential substrate for aspartyl-specific peptidase enzymes and this was considered of interest as synthesis of a number of commercially valuable peptides has been achieved *in vitro* by reversal of proteinase catalysed hydrolysis reactions. Reversal of aspartyl-peptidase catalysed hydrolysis of aspartame would provide a potentially efficient route for the synthesis of this valuable product.

Commercial production of aspartame is currently undertaken using as substrates the N-terminal blocked amino acid, carboxybenzyl-aspartate (CBZ-Asp) and the amino acid ester, phenylalanine methyl ester, in a chemical synthesis of the peptide bond. This is then followed by catalytic dehydrogenation of the derivatised product to form aspartame (Klausner, 1985). This procedure is costly and means that aspartame is commercially more expensive than sucrose and other sweeteners. Major studies

have therefore been carried out to find novel and cheaper methods for aspartame synthesis, some of which have looked at aspartame synthesis by proteinase mediated reversed hydrolysis.

Initial studies on the possible synthesis of peptides and proteins by protease enzymes were aimed at elucidating the mechanisms of protein biosynthesis in living systems (Bergmann & Fraenkel Conrat, 1937; Bergmann & Fruton, 1937). Interest in peptide synthesis by these enzymes faded rapidly when the mechanism of *in vivo* synthesis of proteins was determined during the 1950's. The discovery of new methods for protection of subtrates involved in synthesis, the use of novel solvent systems, and the availability of a wide range of novel microbial proteinase enzymes, led to progress in the use of these enzymes in the production of valuable peptides, including the opioid pentapeptides leu- and Met-enkephalin (Kullmann, 1980) and human insulin (Morihara *et al.*, 1981).

The first experiments aimed at developing proteinase mediated aspartame synthesis used the metalloproteinases thermolysin or prolisin as catalysts for peptide bond formation between N-benzyloxycarbonyl-L-aspartyl-(β-benzyl) and L-phenylalanine methyl ester. The product N-benzyloxycarbonyl-Z-aspartyl-(β-benzyl)-L- phenylalanine methyl ester was produced in high yield (Isowa *et al.*, 1976). The benzyloxycarbonyl group and benzyl groups could easily be removed by catalytic dehydrogenation thus providing a method for manufacturing the

sweetener. Further studies showed that leaving unprotected the side chain carboxyl group of the N-protected aspartate produced an insoluble condensation product which could be converted in a simple process to the sweetener (Isowa et al., 1979).

In an effort to improve the efficiency of this thermolysin catalysed synthesis of peptide, the enzyme was immobilised on an anionic exchange resin and the substrates, N-benzyloxycarbonyl-L-aspartate and phenylalanine methyl ester, incubated with the immobilised enzyme at 40°C in the solvent ethyl acetate. The use of the ethyl acetate solvent instead of water served to push the reaction equilibrium further towards peptide synthesis partly by reducing the amount of water available for hydrolysis, and partly through enzyme-solvent interaction effects, giving improved yields of dipeptide (Oyama et al., 1981).

More recently a system has been described in which N-benzyloxycarbonyl-L-aspartyl phenylalanine methyl ester can be synthesised continuously in an organic solvent using immobilised thermolysin (Nakanishi et al., 1985). This synthesis was run for 300 hours in a stirred tank reactor producing a yield of 90% dipeptide. The solvent used for this synthesis was ethyl acetate.

A number of methods for the enzymic synthesis of aspartame have been patented, most using thermolysin as catalyst (Oshima & Harano, 1987; Snedecor & Hsu, 1986) although other enzymes, for

example a micrococcal protease (Francois et al., 1985) have been used. Though many of these syntheses produce high yields of aspartame, commercial production has been held up due to problems in the isolation and separation of pure product in the solvent systems used (G D Searle pers. comm.).

No reports have been made of attempts to utilise peptidase enzymes for the synthesis of aspartame or other peptides. Heats of formation and equilibrium constants for the synthesis of non-derivatised peptides from amino acid substrates shows that hydrolysis of peptide is highly favoured over synthesis. The equilibrium constant for synthesis of the dipeptide leucyl-glycine from the amino acids leucine and glycine, has been determined at 0.0047 (under standard conditions) and even with initial amino acid concentrations of as high as 0.1M, a level of only 0.04% peptide synthesis was achieved (Borsook, 1953).

Kinetic data was not available for synthesis of the dipeptide aspartame. However it was considered possible that under appropriate conditions peptidase catalysed synthesis of aspartame might produce low levels of dipeptide, and hence be worthy of investigation. Such a synthesis would avoid the need for the derivatisation of aspartate prior to peptide bond formation as well as obviating the need for removal of protecting groups, steps both required in proteinase mediated synthesis. Such a route for aspartame synthesis would bypass the product isolation difficulties confronted in proteinase mediated

synthesis. Experiments aimed at the peptidase catalysed synthesis of aspartame were therefore carried out in this project.

1.8 Project Aims and Strategy

The current investigation aimed originally to find a novel route for the synthesis of the aspartyl-dipeptide sweetener aspartame, by means of peptidase catalysed reverse hydrolysis. It was decided to screen microorganisms both for their ability to utilise aspartame and aspartyl-peptides as sources of required nutrients and for the presence of peptidase activities specific for the hydrolysis of these peptides. Whole cells and crude cell extracts containing peptidase activities were then used in experiments aimed at synthesising aspartame under conditions designed to promote synthesis.

Synthesis of aspartame was not observed under a range of different conditions used. It was therefore decided to concentrate research on a study of the genetics, biochemistry and regulation of the aspartyl peptidases identified in *E. coli* K12. This would provide an insight into the factors involved in aspartyl-peptidase activity. The particular *E.coli* K12 peptidases involved in the hydrolysis of L- α-aspartyl peptides were identified and their subtrate specificities investigated by analysis of genetically constructed *E. coli* K12 strains and by use of gel-based enzyme assays. These peptidases, along with an easily assayable aspartyl-peptidase activity from *Acinetobacter calcoaceticus* were purified and their biochemical properties examined. Genetic and regulatory studies were then carried out in order to further characterise the *E. coli* K12 aspartyl-peptidase enzymes.

CHAPTER 2 MATERIALS AND METHODS

2.1 Amino acids and Peptides

All amino acids used in this work were L-isomers and these were purchased from Sigma Chemicals Ltd., Poole, Dorset. Peptides (all L-form) were purchased from Bachem AG, Bubendorf, Switzerland (Leu-Pro, Pro-Leu, Leu-Leu, Glu-Phe, Asn-Phe, Glu-Phe and all aspartyl peptides except for Asp-PheOMe and Asp-Pro), Research Plus, Bayonne, New Jersey, USA (Asp-Pro), Sigma Chemicals Ltd. (Leu-Gly, Leu-Gly-Gly, Leu-Arg, Leu-Ala-NH_2, Leu-β-napthylamide and Asp-PheOMe) and Fluka AG, Buchs, Switzerland (Phe-Gly-Gly, Phe-Gly, Phe-Leu and Phe-Phe).

2.2 Other Chemicals

The antibiotics streptomycin, tetracycline, psoralen and ampicillin (sodium salt) were purchased from Sigma Chemicals Ltd. as were the nucleotide thymine and vitamin B1 (thiamine). Other chemicals used as buffers and salts in growth media were mostly of Anal R grade and were purchased from BDH Chemicals Ltd., Poole, Dorset or Sigma Chemicals Ltd. Solvents used in thin layer chromatography and peptide synthesis experiments were all Anal R grade while those used for HPLC were HPLC grade. All solvents were obtained from BDH. The amino acid/peptide detection reagents ninhydrin and O-pthalaldehyde were obtained from BDH and Sigma Chemicals Ltd. respectively. Biochemicals used in the qualitative peptidase assay including L-amino acid oxidase (from Croatus Adamenteus Type I) and Horseradish Peroxidase (Type I) were obtained from Sigma Chemicals Ltd. as was the mutagen N-methyl-N'-nitro-nitrosoguanidine (NTG).

Sephadex G200 was obtained from Pharmacia Fine Chemicals Ltd., Poole, Dorset and the anion exchange resin DE52 from Whatmann UK Ltd., Maidstone, Kent. Ammonium sulphate used for protein fractionation and concentration was of Anal R grade.

2.3 Media

2.3.1 Rich Media

Nutrient broth and nutrient agar (Oxoid, Basingstoke, Hampshire) were used as rich media for the growth of bacterial strains and were made up as per manufacturer's instructions. Fungal strains were grown on potato dextrose agar (Oxoid) again made up according to manufacturer's instructions but with an added 0.5% bacteriological agar No. 1 (Oxoid), or for growth in liquid culture, in a medium consisting of the following:

Peptone	5g
Corn Steep Liquor	5g
Sucrose	10g
KH_2PO_4	0.5g
$MgSO_4,7H_2O$	0.5g
$CaCl_2,2H_2O$	1g
dH_2O	$1dm^3$

A solution of trace elements was added at $0.5cm^3/dm^3$ and the medium adjusted to pH 5.5-5.6*, prior to autoclaving at 121°C for 15 minutes. The trace element solution consisted of:

* (pH was adjusted in all media by addition of appropriate volumes of 1N HCl.)

$CuSO_4,7H_2O$	0.4g
$MnSO_4,4H_2$)	2.0g
$ZnSO_4,7H_2O$	2.0g
$FeSO_4,7H_2O$	10.0g
$Na_2MoO_4,2H_2O$	0.5g
dH_2O	$1dm^3$

For the preparation of Phage P1 lysates used in generalised transduction, tryptone agar plates were used consisting of:

Tryptone	10g
Yeast Extract	5g
NaCl	10g
Glucose	10g
Agar	15g
dH_2O	$1dm^3$

The pH of the medium was adjusted to pH 7.2 and the medium autoclaved at 121°C for 15 minutes. When whole bacterial cells were used in experiments aimed at synthesising the dipeptide sweetener aspartame, strains were grown up in an 'aspartame synthesis medium' consisting of:

Malt Extract	5g
Yeast Extract	10g
Glucose	20g
$(NH_4)_2SO_4$	5g
KH_2PO_4	1g

K_2HPO_4	1g
$MgSO_4,7H_2O$	0.5g
$FeSO_4,7H_2O$	0.01g
$MnSO_4,4H_2O$	0.01g
$Ca(CO_3)$0.4g/ml	100 mls
dH_2O	900 mls

and the medium was sterilised at 121°C for 15 mins. $CaCO_3$ was sterilised separately, and the final pH of the medium was adjusted to pH 7.0.

2.3.2 Minimal Media

M9 minimal salts medium was used as minimal medium for the growth of E. coli K12 (Miller, J.H., 1972). This consisted of:

Na_2HPO_4	6g
KH_2PO_4	3g
NaCl	0.5g
NH_4Cl	1g
0.01M $CaCl_2$	10mls
0.1M $MgSO_4,7H_2O$	10mls
Glucose 20%	20mls
dH_2O	160mls

Solutions of $CaCl_2$, glucose, and $MgSO_4,7H_2O$ were sterilised separately. Solutions were sterilised by autoclaving at 121°C for 15 minutes. When solid media was required, 15g of Oxoid bacteriological agar No. 1 was added per litre of medium prior to autoclaving. Unless otherwise stated L-amino acids, peptides and

thymine where required were added to the sterilised salts solution to give final concentrations of 20µg/ml, and where required vitamin B1 was added to give a final concentration of 0.5µg/ml. Streptomycin, tetracycline and ampicillin when required were added at concentrations of 40µg/ml, 50µg/ml and 25 µg/ml respectively and were filter sterilised using either Millipore disposable filters (Millipore, Molsheim, France) or Nucleopore polycarbonate membranes (Sterilin, Teddington, Middx). Thymine and tetracycline were solubilised in 0.1N NaOH and 0.1N HCl respectively prior to sterilisation.

For the growth of B. subtilis BR-17 on minimal medium, the following Spzizen minimal medium was used (Spzizen J., 1958).

$(NH_4)_2SO_4$	2.0g
K_2HPO_4	14.0g
KH_2PO_4	6.0g
$MgSO_4,7H_2O$	0.2g
Sodium citrate	10.0g
Glucose	5.0g
Agar	15.0g

Peptides and amino acids were sterilised separately and added to give a final concentration of 20µg/ml, with the exception of the amino acid tryptophan which was added at 4µg/ml (Barat, 1965).

For growth of fungal strains a general purpose fungal growth medium was used (J.W. Deacon, 1980):

$NaNO_3$	2.0g
KH_2PO_4	1.0g
$MgSO_4,7H_2O$	0.5g
KCl	0.5g
$FeSO_4,7H_2O$	0.01g
$ZnSO_4,7H_2O$	0.01g
$CuSO_4,7H_2O$	0.005g
Sucrose	20.0g
Glucose	20.0g
Agar	15.0g
dH_2O	$1dm^3$

This minimal medium was sterilised by autoclaving at 121°C for 15 minutes.

For experiments in which peptides and amino acids were used as sole nitrogen source, standard nitrogen sources were excluded from media and replaced by amino acids and peptides at the equivalent molar concentration.

2.4 Microorganisms

Strains of E. coli K12 used in this work are listed in Table I. The phenylalanine auxotroph, B. subtilis BR-17 was obtained from Dr J.A. Hoch (Scripps Clinic, La Jolla, California). Fungal strains, initially isolated as producers of high extracellular esterase activity, were provided by Dr Walter Morris (Trent Polytechnic, Nottingham). All other microorganisms

used in this work were obtained from the collection of cultures held at Trent Polytechnic. Phage P1 (*vir*) used for generalised transduction was provided by Dr S.I. Ahmad.

Table I. Strains of *E. coli* K12 used in this work

Strain	Genotype	Source
CM17	*F⁻ leu Δ(pro-lac) met thyA*	C.G. Miller
CM89	*F⁻ leu Δ(pro-lac) met thyA pep A pep B pep N pep Q*	C.G. Miller
KL16	Hfr prototroph	B.J. Bachmann
KA197	*Hfr phe A rel A λ-SpoT1 thi-1*	B.J. Bachmann
AB1157	*F⁻ leu pro his thr thi-1*	B.J. Bachmann
AT2457	*F⁻ glyA rel A λ-SpoT1 thi-1*	B.J. Bachmann
AT3208	*F⁻ pdxJ rel A λ-SpoT1 thi-1*	B.J. Bachmann
JH101	*F⁻ puv^r*	J.H. Holland
SA173	*F⁻ leu*	S.I. Ahmad
SA317	*F⁻ rec A Tn10*	S.I. Ahmad
KT1086	*F⁻ Δ (gpt-proAB-argf-lac) sps L [Mud (Ap^r lac$^+$)] [Mucts 62]*	K.J. Towner
PN1	*F⁻ leu Δ(pro-lac) met thy A Strp^r*	spontaneous strp^r from CM17
PN4	*F⁻ leu Δ(pro-lac) met thy A strp^r pep A pep B pep N pep Q*	spontaneous strp^r from CM89

Strain	Genotype	Origin
PN7	F⁻ leu Δ(pro-lac) met phe A strp^r	Conjugational cross KA197 x PN1
PN10	F⁻ leu Δ(pro-lac) met phe A strp^r pep A pep B pep N pep Q	Conjugational cross KA197 x PN4
PN13	F⁻ leu Δ(pro-lac) met ~~phe~~ A strp^r	Conjugational cross KA197 x PN4
PN16	leu Δ(pro-lac) met phe A strp^r pep B pep N pep Q	P1 transduction CM17 x PN10
PN19	leu Δ(pro-lac) met phe A strp^r pep A pep N pep Q	P1 transduction CM17 x PN10
PN22	leu Δ(pro-lac) met phe A strp^r pep A pep B pep Q	P1 transduction CM17 x PN10
PN25	leu Δ(pro-lac) met phe A strp^r pep A pep B pep N	P1 transduction CM17 x PN10
PN28	leu met phe A strp^r pep A pep B pep N	P1 transduction SA173 x PN10
PN31	asp leu Δ(pro-lac) met phe A strp^r pep A pep B pep N pep Q	Spontaneous asp from PN10

Strain	Genotype	Origin
PN32	asp leu Δ(pro-lac) met phe A strp^r (Asp-Val)^r pep A pep B pep N pep Q	Spontaneous (Asp-Val)^r from PN10
PN53	asp leu Δ(pro-lac) met phe A strp^r pep B pep N pep Q	P1 transduction CM17 x PN31
PN56	asp leu Δ(pro-lac) met phe A strp^r pep A pep N pep Q	P1 transduction CM17 x PN31
PN59	asp leu Δ(pro-lac) met phe A strp^r pep A pep B pep Q	P1 transduction CM17 x PN31
PN62	asp leu Δ(pro-lac) met phe A strp^r pep A pep B pep N	P1 transduction CM17 x PN31
PN65	leu met phe A strp^r pep A pep B pep N pepQ	P1 transduction SA173 x PN31

2.5 Identification of microorganisms

The identity of certain microorganisms was confirmed using a combination of microscopic techniques and API tests.

2.5.1 Microscopy

A modified Gram reaction (Burke, 1922) was used for preliminary identification of bacterial cultures. A small drop of culture was placed on a slide and spread to form a thin film. The slide was dried above a flame and then passed film side up 3 times through the flame. The slide was then flooded with crystal violet to which 3 drops of sodium bicarbonate were added. After 2.5 minutes, the slide was rinsed with tap water and flooded with NaOH/iodine (Grams iodine). After a further 2 minutes the slide was again rinsed with tap water then decolourised with ether/acetone. A further rinse was followed by flooding of the slide with the counter-stain safranin for 10 seconds. After rinsing, the slide was blotted dry and examined under the microscope (Vickers MI5C). Gram positive strains gave a blue/purple colour whereas Gram negative strains were pink/red.

2.5.2 API Tests

The API 20NE test kit for the identification of non-enteric Gram negative rods and the API 20E kit for the identification of enterobacteriaceae and other Gram negative rods were obtained from API Supplies Ltd., Grafton Way, Basingstoke, Hampshire. All identification tests using these kits were carried out as per manufacturer's instructions.

2.6 Maintenance of Microorganisms

Bacterial strains when in regular use were maintained on nutrient agar streak plates at 4°C and these were sub-cultured every month with incubation of newly streaked plates for 48 hrs at 37°C. Otherwise these strains were stored at 4°C on nutrient agar slants and sub-cultured every 3 months. Freshly inoculated slants were incubated at 48 hrs at 37°C before storing at 4°C. For longer term storage some cultures were stored at -20°C in 20% glycerol. 1 ml of sterile 80% glycerol was added to 3 mls of overnight culture grown up in nutrient broth and after vortex mixing, cells were transferred to a -20°C freezer. Fungal strains were grown up on potato dextrose agar slants and after incubation at an appropriate temperature, the slants stored at 4°C.

Cultures were also freeze-dried. The organisms were grown for 24 hrs on nutrient agar slants. The cells were suspended in about 5 mls of sterile skimmed milk and 0.1 ml quantities of the suspension dispensed into cotton wool plug ampoules. The cotton wool plugs were replaced with sterile cloth caps and the ampoules placed in the primary drying assembly of an Edwards Model EF303 freeze-drier. Primary drying, under vacuum was carried out for 2-4 hrs using phosphorous pentoxide as a dessicant. On completion of primary drying, the cloth caps were replaced with sterile cotton wool plugs. These latter were pushed halfway down the ampoules, which were then constricted above the plug. Ampoules were mounted in the secondary drying manifold of the

Edwards freeze-drier. Secondary drying lasted at least 16 hrs after which time, the ampoules were sealed whilst still under vacuum. Lyophilised cultures were stored at 4°C and viability checked by reconstituting the freeze dried cells and plating onto minimal selective plates.

2.7 Determination of Peptide Purity

Peptide homogeneity was confirmed by thin layer chromatography combined with ninhydrin detection. 100 mls of mobile solvent phase, either butanol acetic acid water (4:1:1) or 2-methyl-2-butanol-butanone-propanone-methanol-water-0.88 ammonia (50:20:10:5:15:5), was added to a Shandon S/P TLC tank lined with blotting paper previously soaked in mobile phase. The apparatus was sealed with paraffin wax and left overnight allowing the tank atmosphere to saturate with solvent. Solutions of peptide or amino acid were made up at 1 mM or 10 mM concentration in 10% 2-propanol 10µl samples were then spotted onto 20 cm x 20 cm Cellulose MN300 TLC plates (Anachem, Luton, Beds), 2 cm from the plate edge and after removal of excess solvent in a stream of warm air, the plate was transferred to the pre-prepared TLC tank. When the mobile phase had migrated to about 1 cm from the top of the plate, the chromatogram was removed and sprayed with a 0.1% solution of ninhydrin in acetone, until it appeared translucent. The plate was then heated at 105°C for 15 minutes and cooled at room temperature. Amino acids and peptides appeared as purple or yellow spots on the chromatogram, and their R/f values (distance moved by the amino acid/peptide spot divided by the distance

moved by the solvent front) were recorded.

2.8 Qualitative Growth Tests

2.8.1 Auxotrophic Growth Requirements and Antibiotic Resistance

Growth requirements of amino acid and other bacterial auxotrophs were tested and confirmed by streaking or replica plating individual colonies onto minimal agar plates with and without the appropriate growth factor, then comparing growth after incubation for 48 hrs at 37°C. This method was also used for confirmation of antibiotic resistance properties although in some cases nutrient agar plates containing antibiotic at the appropriate concentration were used.

2.8.2 Utilisation of Peptides as Sole Source of Amino Acid Growth Requirements

The ability of bacterial strains to utilise different peptides as sole source of required amino acids was in some cases tested using the method for amino acid auxotrophs described above. In most experiments, as a means of reducing the expense and amount of peptide used, and also to simplify the test procedure, the following method was followed. A 10 ml overnight culture, grown in nutrient broth, was centrifuged at 4,000 g for 10 minutes on MSE centaur bench centrifuge. The supernatant was removed and the culture resuspended in 5 mls sterile phosphate buffer (0.007% Na_2HPO_4, 0.3% KH_2PO_4, 0.4% NaCl, 0.002% $MgSO_4 \cdot 7H_2O$).

The centrifugation was repeated and the cells resuspended in 0.5 mls phosphate buffer. 0.1 mls of these washed cells was added to 4 mls of 0.6% water agar in a sterile test tube and the resulting mixture overlaid onto a minimal agar plate lacking an appropriate amino acid growth requirement. A few crystals of peptide were placed on the surface of the plate, and the presence or absence of growth scored after 48 hrs incubation at 37°C.

2.8.3 Utilisation of Peptides as Sole Source of Nitrogen

Bacterial and fungal strains were plated out on minimal agar plates containing peptide as sole source of nitrogen. Bacterial colonies were streaked out whereas fungal strains were inoculated by stabbing the medium with a conidia seeded straight wire. After incubation at an appropriate temperature (37°C for bacterial strains; 30°C for fungal strains) for 3-5 days, growth was scored. Minimal agar plates containing amino acids, ammonium or nitrate ions, were used as controls.

2.8.4 Amino Acid Napthylamide Hydrolysis Tests

E. coli K12 colonies were tested for their ability to hydrolyse napthylamide containing peptides after growth on nutrient agar plates (Miller & Mackinnon, 1974). Plates were incubated until colonies were barely visible then stained for enzymatic activity by overlaying with a stain mixture containing 5 ml of 0.2M Tris-HCl pH 7.5, 0.2 ml of amino acid napthylamide solution (10 mg/ml in dimethylformamide) and 10 mg of Fast Garnet GBC (Sigma). Colonies showed a dark red colour, usually in 1-2 minutes if hydrolysis of the peptide had occurred.

2.8.5 Valyl-Peptide Sensitivity Tests

Minimal agar plates were overlaid with a soft agar layer (0.6% water agar) containing 0.2 mls of twice washed and 20-fold concentrated E. coli K12 cells from an overnight nutrient broth culture. 10µl samples of 5 mM peptide were spotted onto the soft agar layer and the plates incubated at 37°C for 48 hrs. The presence or absence of growth inhibition was noted and the diameter of zones of growth inhibition recorded. Samples of the amino acid valine (5 mM) were similarly spotted on soft agar layers for comparison and as controls.

2.8.6 Psoralen-U.V. Light (PUVA) Resistance Tests

10µl samples from overnight nutrient broth cultures of E. coli K12 transductant strains, were spotted on nutrient agar plates containing 25 µg/ml psoralen. Cells were then irradiated at 600j/m^2 for 30 seconds using a Blackray U.V. lamp. Growth was scored after 48 hrs' incubation at 37°C and strains designated PUVA resistant or sensitive.

2.9 Quantitative Growth Tests

For the determination of mean generation times under a variety of different conditions where aspartyl peptides or their constituent amino acids were growth substrates for amino acid auxotrophic strains of E. coli K12, the following general procedures were followed. Initially, strains were streaked out on to minimal agar plates supplemented with appropriate amino acids and after incubation for 48 hrs at 37°C, single colonies

were used to inoculate universal bottles containing 10 mls of minimal medium supplemented with required amino acids. These were incubated at 37°C with shaking at 150 rpm in a Gallenkamp Orbital Shaker. After overnight growth the resulting cultures were centrifuged at 4,000 g for 10 minutes and cell pellets resuspended in 5 mls of phosphate buffer. This washing procedure was repeated with resuspension of cell pellets in 2 mls of phosphate buffer. 0.2 ml samples of these washed cells were then used to inoculate 19.8 mls of appropriately supplemented minimal medium, prewarmed at 37°C in a 100 ml conical flask which was then incubated at 37°C and 150 rpm in the orbital shaker. Immediately after inoculation of the test medium and at regular time intervals during growth, 0.5 ml samples were taken from the flasks and mixed with 0.5 ml dH_2O in 1 ml plastic cuvettes (Kartélle, Divisione Labware). The optical densities ($O.D._{600nm}$) of these samples were determined using a Philips U.V./visible spectrophotometer with a dH_2O control, and the data used for determination of growth rates and mean generation times. These parameters were calculated from the formulae (Stanier et al., 1987):-

$$(1)\ \mu = \frac{(\log_{10} t - \log_{10} t_o)\ 2.303}{T}$$

where μ is the specific growth rate, $t = O.D._{600}$ at the end of the exponential phase, $t_o = O.D._{600}$ at the beginning of the exponential phase and T = time of duration of the exponential phase.

$$(2)\ g = \frac{0.693}{\mu}$$

where g is the mean generation time (doubling time).

Volumes of growth medium were adjusted as appropriate for experiments involving additional growth factors or pH adjustment to give a final volume of 20 mls growth medium in all experiments. pH of the medium was adjusted by addition of 1N HCl or 2N NaOH as appropriate, using a non-sterile control flask of medium to determine required volumes of acid and base.

For determination of lag times (the time before exponential growth phase) for E.coli K12 peptidase mutants, single colonies from nutrient agar plates were used to inoculate 10 mls of nutrient broth in universal bottles. After overnight incubation at 37°C without shaking, 1 ml samples were inoculated into 100 ml conical flasks containing 20 mls nutrient broth, and these flasks were incubated with shaking at 150 rpm and 37°C until cells had reached mid-exponential phase (0.4-0.6). Cells were then washed in phosphate buffer and after resuspension in 0.2 mls of the same, these cells were used to inoculate 19.8 mls prewarmed minimal medium supplemented with amino acids. These cultures were then incubated at 37°C and 150 rpm and growth followed as above.

2.10 Genetic Techniques

2.10.1 Isolation of Streptomycin Resistant Mutants

E. coli K12 cultures were grown up in 40 mls of nutrient broth at 37°C and 150 rpm overnight in an orbital shaker. The

resulting cultures were concentrated by centrifugation and resuspension in 1 ml phosphate buffer. 0.1 ml aliquots were then spread on nutrient agar plates containing 100µg/ml streptomycin (Schlief & Wensink, 1980). After overnight incubation at 37°C resultant colonies were retested for streptomycin resistance by streaking on further streptomycin containing nutrient agar plates. Confirmed streptomycin resistant strains were then tested for the occurrence of other opportunistic phenotypic changes during the isolation procedure.

2.10.2 Bacterial Conjugation

4 ml aliquots were transferred from 10 ml overnight nutrient broth cultures of E. coli K12 Hfr and F^- strains into separate 250 ml flasks containing 20 mls nutrient broth (Miller, 1972). These cultures were incubated in a shaking water bath (Grant) at 37°C until growth had reached mid-exponential phase ($O.D._{600}$ 0.4-0.6).

The cultures were then mixed in the ratio $5F^-$: 1 Hfr in a separate sterile flask and incubated at 37°C with slow aeration. Samples of mating mixture were removed at selected time intervals, diluted where appropriate and 0.1 ml aliquots of mating mixtures and parent cultures, spread on selective plates. In experiments involving interrupted mating, samples of mating mixture were removed and immediately vortexed for 1 minute before plating out. Exconjugant colonies were scored after 48 hrs incubation at 37°C and where appropriate were tested for the presence or absence of additional growth properties.

2.10.3 Generalised Transduction Using Phage P1 (vir)

2.10.3.1 Preparation of P1 (vir) lysates

Generalised transduction was carried out after preparation of an appropriate phage P1 lysate (Miller, 1972). $CaCl_2$ was added to a 10 ml overnight nutrient broth culture of the donor E. coli K12 strain to give a final concentration of 2 mM.0.2 ml aliquots were added to each of 5 sterile test tubes and these were equilibrated for 10 minutes in a 37°C water bath. After equilibration, 0.1 ml of P1 phage was added to 4 of the tubes with the other left as control. After 15 minutes incubation at 37°C, 4 mls of 0.6% water agar kept at 55°C was added to each of the 5 tubes and each mixture overlaid on a tryptone agar plate. These plates were incubated at 37°C until the plates seeded with P1 phage showed confluent lysis of the bacteria in the soft agar layer.

The soft agar layers of the seeded tryptone agar plates were removed and pooled into a sterile 50 ml centrifuge tube. 5 mls of phosphate buffer was added and the contents vortexed for 30 seconds. Following centrifugation at 10,000g and 4°C for 10 minutes using an MSE High Speed Centrifuge, the supernatant (phage lysate) was removed and 10 drops of chloroform added to kill any remaining bacterial cells.

2.10.3.2 Titration of Phage P1(vir) lysates

For phage titration, phosphate buffer* was used to prepare 10^{-5}, 10^{-6} and 10^{-7} dilutions of the phage lysate. An E. coli

* phosphate buffer as P.72

K12 recipient strain was infected with 0.1 ml of each phage dilution, as described above. After incubation of overlaid tryptone agar plates for 24 hrs at 37°C, phage plaques were counted and the phage titre determined.

2.10.3.3 Genetic Transduction Using P1(*vir*)

2 x 10 ml overnight nutrient broth cultures of recipient strain were harvested by centrifugation at 4,000g for 10 minutes using an MSE bench centrifuge. After removal of the supernatant, the cell pellets were resuspended in 5 mls of fresh nutrient broth and $CaCl_2$ added to give a final concentration of 2 m*M*. Titred phage P1 was added to one culture tube at a multiplicity of infection of 1 (the other recipient culture being used as a control), and the infected and uninfected cultures incubated at 37°C for 15 minutes. Both cultures were then centrifuged at 4,000g for 10 minutes and the cell pellets resuspended in 1 ml phosphate buffer supplemented with 0.25% sodium citrate (to prevent further phage infection). 0.1 ml aliquots from each tube were spread on appropriate selective plates and transductants scored after incubation at 37°C for 48 hrs. In genetic mapping experiments transductant colonies were further tested for the development of unselected growth phenotypes.

Map distances between different markers on the *E. coli* K12 chromosome were determined from co-transduction frequencies using the formula

$$\text{Co-transduction frequency} = (1 - \frac{d}{2})^3$$

where d is the genetic map distance in minutes (Wu 1966).

2.10.4 Mutagenesis with Phage Mud I (lac, Ap)

Lysate preparation and transduction with phage Mud I (lac, Ap) was carried out as previously described (Silharvy et al., 1984) except that phage lysates were prepared from the strain E. coli KT1086 (lac, Ap).

2.10.4.1 Preparation of Mud I (lac, Ap) Lysates

A single colony of the Mud I (lac, Ap) lysogen KT1086 was used to inoculate 5 mls of nutrient broth in a culture tube which was then incubated overnight at 30°C and 150 rpm in a rotary shaker. 0.05 ml of this overnight culture was then used to inoculate a further 10 mls of medium which was incubated at 30°C with aeration until the cells reached early log phase ($O.D._{600}$ 0.15). This culture was then incubated at 50°C for 30 minutes in a shaking water bath, and after this time, incubated at 37°C with shaking until the cells had lysed. 0.1 ml of chloroform was added and after vortexing, this lysate was centrifuged at 4,000g for 10 minutes to pellet debris. The resulting clear lysate was transferred to a sterile screw-capped bottle and a further 0.1 ml of chloroform added then the tube vortexed. This mud lysate was stored at 4°C.

2.10.4.2 Transduction with Mud I (lac, Ap)

A single colony of recipient strain was used to inoculate 5 mls of nutrient broth in a culture tube and this was incubated at 37°C and 150 rpm overnight. The overnight culture was centrifuged at 1,500g for 10 minutes and the cell pellet resuspended in 2.5 mls of 10 mM $MgSO_4.^{7H_2O}$ containing 5 mM $CaCl_2$. The Mud (lac, Ap) lysate was diluted to 10^{-2} and 10^{-3} in phage dilution buffer (0.12% Tris, 0.12% $MgSO_4$, Gelatin 0.01%, pH 7.4 with HCl). 0.1 ml recipient cells were mixed with 0.1 ml of each lysate dilution and cell only and lysate only controls prepared. The contents of the tubes were gently agitated and incubated for 20 minutes at 30°C without shaking. 0.2 ml aliquots of each mixture were plated on nutrient agar plates containing 25 µg/ml ampicillin and these plates incubated at 30°C for 48 hrs. Colonies were then tested for their resistance to the dipeptide Asp-Val, or their ability to utilise aspartyl-peptides as source of required amino acids by replica plating onto peptide and amino acid containing plates using sterile toothpicks. Alternatively a microassay screen was employed to select for aspartyl peptidase deficient mutants.

2.10.4.3 Microassay Screen for Aspartyl Peptidase Deficient Mutants

Attempts were made to identify mutants with reduced ability to hydrolyse aspartyl peptides by screening clones using a peptidase microassay (Carter & Miller, 1984). Selected clones were transferred from agar plates to wells of plastic depression

plates (Sterilin) containing 200 µl M9 medium supplemented with appropriate amino acids. These plates were incubated at 30°C and replica cultures for storage inoculated onto nutrient agar plates. Cells in the depression plate were harvested by centrifugation, washed once in 100 µl of 0.1M Tris-HCl (pH 7.5) and resuspended in 20 µl of lysis buffer (1 mg/ml lysosyme, 5 mM EDTA in 0.1M Tri-HCl pH 7.5). Lysis was accomplished by 3 cycles of freezing (-70°C) and thawing. Substrate solution (90 µl of 5 mM Asp-Leu in 0.1 M Tris-HCl pH 7.5, 1 mM EDTA) was added and the plates were incubated at room temperature for 1 hr. Hydrolysis of the peptide was detected by adding 20 µl of a solution containing 0.7 mg of L-amino and oxidase/ml, 1.4 mg horseradish peroxidase/ml, and 0.7 mg O-dianisidine/ml in 0.1 M Tris-HCl pH 7.5 and observing the development of an orange brown colour after 1 hr at room temperature.

The aspartyl-peptidase microassay was also used as a preliminary screen for aspartyl peptidase activity in fractions obtained from gel filtration and ion-exchange chromatography. 50 µl samples of isolated fractions were added to peptide substrate mixtures in the absence of EDTA, and the assay continued as above with observation for the orange-brown colour indicating the presence of enzyme activity.

2.10.4.4 Selection of Aspartyl-valine Resistant Mutants

10 ml overnight cultures of an E. coli K12 peptidase mutant strain grown up on nutrient broth, were centrifuged at

4,000g for 10 minutes and the resulting cell pellet resuspended in 1 ml phosphate buffer. 0.1 ml aliquots were overlaid on minimal agar plates containing 10^{-4} M Asp-Val. Colonies showing growth after incubation for 48 hrs at 37°C were replica plated onto 10^{-4} M valine minimal plates, and 10^{-4} M Asp-Val dipeptide containing plates using sterile toothpicks. Colonies showing growth on 10^{-4} M Asp-Val but not 10^{-4} M valine plates were subjected to further growth or biochemical tests. Cells mutagenised with NTG or with phage Mud I (Ap, lac) after initial selection for Asp-Val resistance were also replica plated onto 10^{-4} M Asp-Val and 10^{-4} M Val plates, and $(Asp\text{-}Val)^r$ mutants subjected to further testing. In some experiments higher concentrations of Asp-Val were used for initial selection and subsequent tests.

2.10.5 Mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine (NTG)

E. coli K12 cultures were grown up overnight in minimal medium and the following day sub-cultured into a further 20 mls of fresh minimal medium in a 250 ml conical flask. Cells were incubated at 37°C in a shaking water bath until mid-exponential phase (O.D. 0.4-0.6) then harvested by centrifugation and resuspended in citrate buffer (0.1 M sodium citrate pH 5.5). The mutagen, NTG, was added to a final concentration of 50 μg/ml and the cells mutagenised for 30 minutes (Schlief & Wensink, 1980). Cells were then washed twice in 'NTG phosphate buffer' (0.1 M KH_2PO_4/K_2HPO_4, pH 7.0) and NTG containing buffer discarded. Cells were

then spread on Asp-Val dipeptide containing plates and colonies resistant to this dipeptide isolated.

2.11 Manufacture of Bacterial Cell-Free Extracts

For small scale manufacture of bacterial cell free extracts, 200 ml cultures were grown up at 37°C in nutrient broth. Cells were harvested in early stationary phase ($O.D._{600}$ = 1.2) by centrifugation at room temperature at 6,000g for 10 minutes. All subsequent operations were carried out at 4°C to minimise enzyme denaturation. Cells were washed once with 50 mls ice-cold 0.05 M Tris-HCl pH 7.5 0.1M KCl, centrifuged as above and resuspended in 4 mls 0.05M Tris-HCl pH 7.5. Cell suspensions were sonicated for 45 seconds using an MSE 100W disintegrator. Unbroken cells and cell debris were removed from the sonicate by centrifugation at 38,000g for 15 minutes in the 8 x 50 ml rotor of an MSE High Speed 18 centrifuge. The resultant clear supernatant was decanted and dialysed overnight at 4°C against 0.05 M Tris-HCl pH 7.5 The dialysed cell extracts were stored at -20°C until required. The cell extract from an unclassified 'Rhizobium' was provided by Dr J A Leigh.

For large scale production of cell-free extract required for enzyme purifications 10dm^3 cultures were grown up in 5 x 3dm^3 shake flasks and harvested as above. Cells were washed once with 200 mls 0.05 M Tris-HCl pH 7.5 0.1 M KCl then resuspended in 20 mls 0.05 M Tris-HCl pH 7.5. These washed cells were transferred to the cylinder of a French Pressure Cell which had previously

been cooled at 4°C for 1 hr. Pressure was applied from a hydraulic press at 1000lbs p.s.i. and the eluant containing broken bacterial cells was collected. Unbroken cells and cell debris were removed and the preparations decanted, dialysed and stored as above.

2.12 Manufacture of Fungal Cell Free Extracts

Conidia were inoculated into rich medium (200 mls) and incubated with shaking for 48 hrs at 30°C. The mycelial mass was filtered and washed with 50 mls 0.05 M Tris-HCl pH 7.5, then resuspended in 5 mls of the same. The mycelium was homogenised at top speed in a Waring homogeniser at 4°C. The cell homogenate was centrifuged at 6,000 rpm and 4°C, and this process was repeated 3 times with the pellet removed on each occasion. The resulting supernatant was dialysed overnight against 0.05 M Tris-HCl pH 7.5. Dialysed supernatants were stored at -20°C.

2.13 Preparation of Media for Extracellular Peptidase Assays

Growth medium produced during fungal extract preparation or generated as supernatant after centrifugation of bacterial cultures, was collected and freeze dried using an Edwards Mini-Fast 680 freeze drier. Freeze dried medium was resuspended in 5 mls 0.05 M Tris-HCl pH 7.5 and this suspension dialysed overnight against the same buffer. Dialysed supernatants were stored at -20°C.

2.14 Estimation of Protein

Protein was estimated by a colorimetric method involving the formation of copper-protein complexes (Lowry et al., 1951; Schlief & Wensink, 1980).

Samples containing 10-100 µg soluble protein were made up to 0.5 ml with distilled water. On the day of the assay, estimation reagent was made up containing

2 ml of 2% (w/v) $CuSO_4$

2 ml of 4% (w/v) Sodium Potassium Tartrate

98 ml of 3% (w/v) Na_2CO_3 in 0.1N NaOH

5.0 mls of this reagent was added to each sample and after mixing, the reagent/sample mixtures incubated at room temperature for 10 minutes. After this period, 0.5 mls of a 1:1 Folin-Ciocalteu reagent: water solution was added and the solution mixed vigorously for 30 seconds. After an additional 30 minutes at room temperature, the absorbance of each sample was measured at 650 nm using the Philips PU 8600 Visible/UV spectrophometer, against a reagent blank containing no protein. A standard curve was prepared by assaying dilutions of a 10 mg/ml stock solution of bovine serum albumen.

Protein was also estimated in gel filtration and ion-exchange fractions by measuring absorbances at 280 nm, samples being transferred to quartz cuvettes then analysed using the Philips spectrophotometer.

2.15 Concentration of Proteins

Concentrated preparations of crude cell-free extracts for analysis on polyacrylamide gels were prepared by ammonium sulphate precipitation. 1 ml of cell extract was transferred to an eppendorf tube held in an ice-water bath. 0.46g ammonium sulphate (giving 65% saturation) was added over a period of 10 minutes with intermittent vortexing of the mixture. The mixture was kept on ice for 40 minutes with frequent vortexing to aid protein precipitation. The eppendorf was then centrifuged at 10,000 rpm for 20 minutes in a microfuge, the supernatant removed and the protein pellet resuspended in 200 µl 0.01 M Tris-HCl pH 7.5 giving approximately a 5-fold increase in protein concentration. The concentrated cell extract was dialysed against 0.01 M Tris-HCl pH 7.5 and the precise protein concentration subsequently determined.

During the large scale purifications of peptidases from *E. coli* K12 and *A. calcoaceticus*, proteins were concentrated at appropriate stages by ammonium sulphate fractionnation or over an ultrafiltration membrane (Centricon-10 Amicon Corp.) used as per manufacturer's instructions.

2.16 Polyacrylamide Gel Electrophoresis of Cell-Free Extracts

Cell extract and partially purified peptidase enzymes were separated by disc polyacrylamide gel electrophoresis (Davis, 1964). The polyacrylamide separation gels (7.5% w/v) were prepared from the following:

3.00 ml of 76 mM Tris/glycine buffer pH 8.3
containing 0.17% (w/v) tetramethylenediamine (TEMED)

2.25 ml of 33% (w/v) acrylamide

0.50 ml of 2.25% (w/v) methylene-bis-acrylamide

3.25 ml of distilled water

The above solutions were mixed and air removed from the solution by evacuation in a Buchner flask for 2 minutes. A 0.1 ml volume of freshly prepared 0.75% (w/v) ammonium persulphate solution was added to initiate gel polymerisation, and after gentle mixing the resultant solution was dispensed into stoppered gel tubes (5 mm internal diameter), filling to within 1 cm of the top of the tube. Distilled water was layered onto the gel surface to present oxygen absorption and meniscus formation. After the completion of gel polymerisation, distilled water was removed from the gel surface, the gel tubes unstoppered and placed in the electrophoresis tank (Pharmacia). The tank was filled with 38 mM Tris/glycine buffer pH 8.3 containing 0.085% (w/v) TEMED, ensuring that both ends of the gels were adequately covered by the solution.

Cell free extracts were diluted to 4-6 mg/ml and sucrose and bromophenol blue added to give final concentrations of 10% (w/v) and 0.001% (w/v) respectively. Cell-free extract (50 µl) was applied to the top surface of each gel, using a Hamilton 50 µl syringe, and immediately overlaid with 25 µl 5% (w/v) sucrose.

The tank was then connected to the power supply (Pharmacia EPS 400/500) and electrophoresis performed at 4°C using a current of 1mA per gel tube until samples had entered the gels, then 3mA per gel tube. Care was taken to ensure that the voltage did not exceed 150V during electrophoresis to avoid undue heat formation and resultant denaturation of the proteins within the gels. Electrophoresis lasted 45-60 minutes, the power supply being disconnected when the bromophenol blue marker had reached to within 5 mm of the gel surface. Gels were immediately removed from their tubes and stained for peptidase activity.

2.17 Staining of Polyacrylamide Gels

2.17.1 Peptidase Activity Staining

Peptidase activities were detected as bands of orange-brown colour in polyacrylamide gels (Lewis & Harris, 1967; Miller & Mackinnon, 1974). The reaction mixture was prepared by dissolving 5 mg of peptide in 2 mls of 0.2 M phosphate buffer pH 7.5 containing 0.5 mg of L-amino acid oxidase (Type I), 0.8 mg of Horseradish Peroxidase (Type I), 0.08 ml of an aqueous solution of O-dianisidine (5mg/ml) and 0.045 ml of 0.1 M $MnCl_2$. This solution was then mixed with an equal volume of 2% agar (at 50°C) and the resulting solutions poured into a test tube containing the gel. Orange-brown bands of activity appeared after 10-60 minutes of incubation at room temperature. Mobilities of the peptidase activity-bands were determined relative to the distance moved on the gel by the bromophenol blue markers.

2.17.2 Staining for Amino Acid Napthylamide Hydrolytic Activity

Amino acid napthylamide hydrolysing activities were identified as dark red bands on polyacrylamide gels (Murgier et al., 1976). Gels were incubated in a mixture containing 0.1 ml of a dimethylformamide solution of the napthylamide (10 mg/ml) and 10 mg of fast garnet GBC in 5 ml of 0.2 M Tris-HCl pH 7.5. Mobilities of bands were determined as for peptidase activity strains.

2.17.3 Protein Staining

Polyacrylamide gels were stained for protein after electrophoresis (Laemmli 1970). Protein was first fixed in the gel by soaking in a solution consisting of 5 volumes methanol: 1 vol acetic acid: 5 vols H_2O. After 1 hr of fixing gels were stained for 10-15 minutes in fixative solution containing 0.1% Coomassie Blue. Destaining of gels was then carried out using a 50 vols methanol: 70 vols acetic acid: 880 vols H_2O solution; the gels were destained for as long as necessary (>18 hrs). Proteins were visible as blue bands on the gels.

2.18 Quantitative Assays for Peptidase Activity

Peptidase activity was routinely assayed in 1 ml reaction volumes containing 3 mM dipeptide in 0.1 M Tris-HCl pH 7.5 and 0.005 units enzyme activity (0.4 mg/ml protein from crude cell extracts). Metal ions and inhibitors were included in the reaction medium as required. pH and substrate concentrations

were adjusted as necessary. In standard assays, incubation of reaction mixtures was carried out for 30 minutes at 37°C. 50 µl of 1.2 M trichloroacetic acid (TCA) was then added to stop the reaction and precipitate protein. After centrifugation at 10,000g for 10 minutes in the microfuge, the supernatant was removed. In the case of TLC based assays this supernatant was directly assayed but for analysis by reversed-phase high pressure liquid chromatography (HPLC) this supernatant was neutralised by addition of 100 µl 2N NaOH, in preparation for derivatisation with O-pthalaldehyde (OPA). In time course assays 100 µl samples were removed from the reaction mixture and the reaction stopped by addition of 5 µl TCA. The reaction was found to be linear over the period of 30 minutes used for assays and the rate of enzyme catalysed hydrolysis under these conditions was proportional to protein concentration (0.1-0.5 mg ml^{-1}) and enzyme levels (0.1-0.005 units ml^{-1}) when purified E. coli K12 peptidases and crude cell extracts were used in assays. No enzyme controls were used in all peptidase assays. One unit of enzyme activity was defined as the amount of enzyme that catalyses the hydrolysis of 1 µmol peptide per minute under standard conditions.

2.18.1 Quantitative Determination of Aspartyl Peptidase Activity using Thin Layer Chromatography

In initial experiments aimed at estimating the level of asparty-peptide hydrolysing activity in microbial cell extracts, an assay based on thin layer chromatography and ninhydrin

detection was used (Clark, 1968). 10 µl samples of assay mixture were spotted onto a 10 cm x 10 cm cellulose thin layer plate (Anachem) using a micropippette, and after the spots were dried in a hot air stream the chromatogram was run in butanol-glacial acetic acid-water (4:1:1) by ascending chromatography (see section 2.7). After removal from the TLC tank, the chromatogram was sprayed with a 2% solution of ninhydrin in ethanol and after all the ethanol had been evaporated off using a hot air stream, the plate was developed in a dark cupboard overnight. Each resulting spot was outlined with a blunt pencil and identified. The spots were scraped off and placed in a 1.2 x 10 cm test tube and 2 mls 50% propyl alcohol in distilled water was added. Elution required 20 minutes after which each tube was shaken vigorously and left for a further 20 minutes to allow the cellulose particles to settle. The optical density at 570 nm of each eluant solution was determined, as well as for blanks after setting the spectrophotometer at 100% transmission for 50% propyl alcohol. The optical density of the appropriate blank was then substracted from the optical density of eluant fractions, and the extent of hydrolysis determined by comparison with a standard curve of aspartate concentration standards eluted from TLC plates, against $O.D._{570}{}^{nm}$.

2.18.2 Quantitative Determination of Peptidase Activities using Reversed Phase HPLC

A wide range of peptidase assays were carried out with both crude and purified peptidase preparations, where reaction

products were assayed using reversed phase HPLC combined with pre-column derivatisation of reaction mixtures with O-pthalaldehyde (OPA) and post-column fluorimetric analysis (Price et al., 1984).

2.18.2.1 Pre-Column Derivatisation of Reaction Mixtures with O-pthalaldehyde

Immediately before use, 250 µl of OPA reagent (60 mg/10 mls methanol) was added to 250 µl 0.4 M boric acid/KOH buffer pH 9.4 and 25 µl β-mercaptoethanol. The mixture was vortexed for 10 seconds then 250 µl removed and added to 12.5 µl enzyme assay sample mixed with 12.5 µl of 0.1 mM taurine internal standard. After vortex mixing for 1 minute, 50 µl of the derivatised mixture was loaded onto the HPLC system sample loop using a Hamilton syringe.

2.18.2.2 Reversed-Phase High Pressure Liquid Chromatography

Derivatised components of the loaded samples were separated on a Zorbax C_8 high speed column (DuPont Instruments 'Golden Series' 8cm x 6.2mm internal diameter, particle size 5 µm) after passage through a 6 cm x 39 mm internal diameter guard column (30.38 µm particle size). Samples were eluted using a gradient formed by mixing 2 solvents: solvent A consisting of 0.02 M pottasium acetate buffer, pH 5.5 containing tetrahydrofuran (1% v/v) and solvent B consisting of methanol containing tetrahydrofuran (1% v/v). Prior to use both solvents

were vacuum filtered through a 0.2 µm nitrocellulose filter (Millipore) then degassed under vacuum. The gradient of solvents was programmed with a DuPont 8800 gradient controller and was run through the column using a DuPont 8800 HPLC chromatographic pump at a flow rate of 1.8 ml min^{-1}. The gradient itself was non-linear running from an initial level of 20% solvent B (maintained for 2.5 minutes), from 20% up to 41% B (over 5 minutes) maintained at this level for 2.5 minutes, and finally run from 41% up to 90% B (10 minutes). The gradient was returned to start conditions before loading further samples.

2.18.2.3 Post-Column Detection of Fluorescent O-pthalaldehyde Derivatives

Fluorescence was measured by means of a Perkin-Elmer model 1000 fluorescence detector fitted with a 25 µl flow cell, a 339 nm interference excitation filter and an emission wavelength of 455 nm. Chromatograms were plotted and integrated by means of a Spectra-Physics model 4100 computer integrator. Peak areas were standardised by comparison with the taurine internal standard, and moles of amino acid present determined from standard curves of moles amino acid plotted against peak areas.

2.19 Assay for Aspartyl-ß-napthylamide (ANA) Hydrolysing Activity

ANA hydrolysing activity was routinely assayed in reaction mixtures containing 1mM ANA (Miller & Mackinnon, 1974). 12.8 mg of ANA was dissolved in 0.5 ml 96% ethanol then diluted to a

volume of 25 mls with 0.05 M Tris-HCl pH 7.5. Large particles of ANA left out of solution were broken up and dissolved by sonication. Enzyme solution was added to give a final reaction volume of 1.0 ml. The reaction was followed in a spectrophotometer cuvette held in the 37°C heated chamber of a Beckmann Du7 spectrophotometer by monitoring the change in optical density at 340 nm and initial reaction velocities were determined (ξ = 1.780 $mM^{-1}cm^{-1}$). A no enzyme reference blank was used. One activity unit in these assays was defined as the amount of enzyme required to hydrolyse 1 µmole min^{-1} of ANA under standard conditions. Metal ions, pH, inhibitor and substrate concentrations were varied as required.

2.20 Purification of Bacterial Peptidases

2.20.1 Ammonium Sulphate Fractionation

In preliminary experiments aimed at the identification of ammonium sulphate fractions containing peptidase activities, 1.95g ammonium sulphate was added over a period of 10 minutes to 10 mls of French Pressure Cell generated cell-free extract held in an ice-water bath. This 28% saturated extract was stirred for a further 20 minutes on ice then the resulting precipate removed by centrifugation at 10,000g for 20 minutes and resuspended in 2.5 mls 0.05 M Tris-HCl pH 7.5. The supernatant was retained and a further 0.25g ammonium sulphate added to the ice cooled supernatant giving 34% saturation. After stirring on ice for a further period of 20 minutes the precipitate formed was removed by centrifugation and resuspended in 2.5 mls 0.05 M Tris-HCl pH

7.5. The same procedure was used with the supernatant generated, to create a 40% saturation level (addition of 0.42g ammonium sulphate) then a 60% saturation level (addition of a further 1.41g) with precipitates removed and resuspended as above. All fractions were dialysed overnight at 4°C against 0.05 M Tris-HCl pH 7.5 to remove residual ammonium sulphate, then assayed for peptidase activities.

Following identification of peptidase activity containing fractions, 50 ml samples of French Pressure Cell generated extracts were subject to fractionation using the ammonium sulphate concentrations in the range 40-60%, by addition of 13.0g ammonium sulphate, removal of protein precipitate then addition of a further 7g ammonium sulphate. The resultant pellets were retained and resuspended in 10 mls 0.05 M Tris-HCl pH 7.5 prior to dialysis to generate an ionic strength suitable for subsequent ion-exchange chromatography and ro remove ammonium sulphate. These 40-60% fractions were assayed for peptidase activity.

2.20.2 Ion Exchange Chromatography

A Pharmacia glass chromatography column (1.5cm x 50cm) was packed using a slurry of DEAE-cellulose (Whatmann DE52) prepared in 0.05 M Tris-HCl pH 7.5 buffer, according to the manufacturer's instructions. The same buffer was used to equilibrate the packed column until the pH of the starting buffer and column eluant were identical (= 2 column volumes of buffer).

Dialysates obtained from ammonium sulphate fractionation were applied via a 3-way tap to the top of the column bed and elution

commenced using 0.05 M Tris-HCl pH 7.5 as starting buffer. The rate of buffer flow through the column was adjusted to 24 mls hr^{-1} and remained constant throughout the elution. 0.5 M NaCl was used to create a linear gradient of increasing ionic strength of 500 ml total volume. The eluant was collected in a Redi-rac automatic fraction collector (LKB Instruments Ltd., Croydon, Surrey). 6 ml fractions were collected every 15 minutes and after collection protein levels were estimated by measuring the extinction at 280 nm using the Philips spectrophotometer. Fractions were screened for peptidase activity using the qualitative microassay screen, and activity containing fractions assayed quantitatively by HPLC based assays. Pooled peak activity fractions were concentrated for gel filtration chromatography by ultrafiltration and after resuspension in 0.05 M Tris-HCl pH 7.5 enzyme solutions were stored at -20°C until required.

2.20.3 Gel Filtration Chromatography

A slurry of Sephadex G200 (fine grade) was prepared in 0.05 M Tris-HCl pH 7.5 buffer and degassed according to manufacturer's instruction. A Bio-rad chromatography column of internal dimensions 15 mm (diameter) x 1,000 mm (length) was packed with the slurry (giving a 170ml column volume, V_t) and the gel washed with the above buffer, using a constant flow rate of 6 ml hr^{-1} until a constant bed volume was achieved.

Concentrated peptidase containing fractions from ion exchange chromatography was applied to the top of the gel bed and eluted with 0.05M Tris-HCl pH 7.5. 1 ml fractions of the column effluent were taken at 10 minute intervals using a Redi-rac automatic fraction collector and protein monitored at 280 nm.

Fractions were assayed for aspartyl peptidase activity qualitatively using the microassay screen and quantitatively after HPLC. Fractions containing activity were pooled and concentrated by ultrafiltration and after dialysis stored at -20°C until required for further studies.

2.21 Molecular Mass Determinations

2.21.1 Molecular Mass Estimation of Partially Purified Peptidases by Gel Filtration

The molecular mass values for partially purified peptidase enzymes were estimated using gel filtration. A column of Sephadex G200 was prepared as described above and the column void volume (V_o) and maximum retention volume (V_e) determined, using solutions in elution buffer of Blue dextran (2 mg/ml) and potassium dichromate (2 mg/ml) respectively. Solutions of pure proteins from a Pharmacia Molecular weights marker kit were then applied to the column and their elution volumes (V_e) determined. The molecular weight markers used were catalase (M_r = 232 kd), aldolase (M_r = 158 kd), bovine serum albumen (M_r = 67 kd), chymotrypsinogen A (M_r = 25.4 kd) and ribonuclease A (M_r = 13.7 kd). The elution volumes for partially purified peptidase

enzymes were determined after loading of concentrated peak activity fractions from ion-exchange chromatography, and their approximate molecular weights determined from a calibration graph of V_e/V_o against $\log_{10} M_r$ for the marker proteins, the line of best fit being generated by least squares analysis.

2.21.2 Molecular Mass Determination of Partially Purified Peptidases on Non-Denaturing Polyacrylamide Gels

Molecular mass values were also estimated by analysis of peptidase mobilities on different concentration PAGE gels (Smith & Hedrik, 1968). Non-denaturing polyacrylamide disc gels were made up at 6.0%, 8.0%, 10%, 12% and 15% acrylamide using appropriate volumes of 33% acrylamide, 2.25% Met-bis-acrylamide, 76 mM tris/Glycine buffer pH 8.3 containing 0.17% (w/v) TEMED, dH_2O and 0.75% ammonium persulphate for polymerisation. Molecular weight markers at 4 mg/ml mixed with bromophenol blue were applied to the top surface of differing concentration gels which were run as described above (Section 2.16). Mobility values were determined after protein staining for each molecular weight marker, as well as peptidase activity staining for peptidase enzymes on each different concentration gel. Plots of mobility against % acrylamide concentration were drawn for both standards and peptidase enzymes and retardation coefficients (K_r) as represented by the slope of each protein standard graph were plotted against molecular mass. From the standard curve produced, the molecular mass values of aspartyl-peptide hydrolysing enzymes were determined.

2.22 Methods used for the attempted Synthesis of the Aspartyl Dipeptide Aspartame

2.22.1 Synthesis Methods using whole Bacterial Cells

Bacterial strains were grown up overnight in 50 mls of 'aspartame synthesis medium' (section 2.3.1) at 30°C in a 500 ml conical flask shaken at 150 rpm (Ajinomoto Corp. 1985). The bacterial cells were harvested by centrifugation and washed with 50 mls 0.85% sterile saline. The collected bacteria were then added to 5 mls of incubation buffer (L-aspartate 2 mM, L-phenylalanine methyl ester 4 mM, in 0.01 M phosphate buffer final pH 5.4) at a concentration of 0.2-2.0g/100 mls, and incubated for 16/24 hrs at 30°C. After incubation, cells were harvested by centrifugation and the supernatant analysed by thin layer chromatography combined with ninhydrin detection for the presence of the aspartame dipeptide.

2.22.2 Synthesis Methods using Crude Bacterial Cell Extracts and Purified Peptidase Preparations

Experiments were carried out using crude cell extracts from bacterial strains and partially purified bacterial aspartyl-peptide hydrolysing enzymes in an effort to synthesize aspartame. Synthesis reactions were performed in 1 ml or 10 ml reaction volumes containing from 0.02-0.05 enzyme activity units, aspartate and phenylalanine/phenylalanine methyl ester substrates at concentrations ranging from 2-10 mM, and in a number of different solvent systems all containing aqueous Tris-HCl buffer. Where water-miscible organic solvents were used, substrates were

dissolved in the water-organic solvent mixture, the hydrogen ion concentration adjusted as required by addition of HCl and enzyme activity containing fractions added to the reaction mixture with continuous stirring. In experiments using the water immiscible co-solvent ethyl-acetate, substrates were dissolved in the aqueous Tris-HCl buffer at appropriate pH and enzyme activity containing fractions added to the aqueous layer. This aqueous layer was then added to the ethyl acetate solvent.

Incubations were carried out for periods of up to 24 hours at 30°C with reaction mixtures agitated using a shaking water bath. At the end of the incubation period samples of aqueous phase, aqueous-organic miscible solution, or organic phase were taken from the incubation mixtures. Water containing samples were directly analysed for the presence of aspartame using TLC combined with ninhydrin detection and the solvent 2-methyl-2-butanol-butanone-propanone-methanol-water-0.88 ammonia (50:20:10:5:15:5) as mobile phase (section 2.7). The ethyl acetate organic phase samples were evaporated to dryness under vacuum and the resultant solid resuspended in 10% 2-propanol. This sample was then analysed for the presence of aspartame using TLC as above.

In some cases aqueous phase samples were analysed for the presence of aspartame using reversed-phase HPLC. 1.2M TCA was added to sample mixtures to precipitate protein and after centrifugation and neutralisation of product mixture, these were derivatised with OPA (section 2.18).

CHAPTER 3 STUDIES ON THE MICROBIAL UTILISATION AND DEGRADATION OF L-α-ASPARTYL PEPTIDES

3.1 Introduction

This particular experimental section of the project was aimed at identifying microorganisms which could use L-α-aspartyl peptides, in particular the dipeptide sweetener aspartame (Asp-PheOMe), as sources of nutritional requirements. In addition, as it was known that peptide hydrolysis to amino acids is a prerequisite for peptide utilisation, it was decided to identify peptidase enzymes involved in the hydrolysis of L-α-aspartyl peptides in different microbial systems. The work on this section focuses in particular on the utilisation and degradation of these peptides in the bacterium Escherichia coli K12.

It has already been mentioned in the general introductory section that peptides of appropriate amino acid composition can usually substitute for free amino acids in satisfying the growth requirements of amino acids auxotrophs, and that some peptides have been found to serve as sole sources of nitrogen in certain microorganisms. This means that quantitative and qualitative growth studies can be carried out by monitoring microbial growth responses on exposure to peptides under appropriate conditions. These principles form the basis of the growth tests used in this section, in which L-α-aspartyl peptides serve as sole source of required amino acids or nitrogen. On account of the lengthy procedures and general difficulties involved in the successful isolation of amino acid auxotrophs from the many

microorganisms considered here, tests for aspartyl peptide utilisation with all microorganisms except for the bacterial strains E. coli K12 and B. subtilis BR-17, were carried out using these peptides as sole sources of required nitrogen.

E. coli K12 has been thoroughly studied and its genetics, biochemistry and physiology are more completely understood than those of any other microorganism. The ability of E. coli K12 to use particular peptides as sources of required amino acids has been the subject of several investigations (Miller & Schwartz, 1978; Simmonds & Fruton, 1949) and peptidase mutants carrying various amino acid auxotrophic markers were consequently available for use in the studies carried out in this project. The use of genetic techniques to extend the auxotrophy of these particular E. coli K12 strains, through acquisition of additional amino acid markers, would broaden the range of peptides that might be used in nutritional studies. In particular the construction of either an aspartate or phenylalanine auxotroph would facilitate studies of E. coli K12 aspartame (Asp-PheOMe) utilisation. The construction of appropriate phenylalanine auxotrophs was carried out using bacterial conjugation between an appropriate E. coli K12 Hfr (High frequency of transfer) phenylalanine auxotroph and appropriate peptidase parent and mutant strains.

The genetic technique of generalised transduction mediated by the bacteriophage P1*vir* was used to construct *E. coli* K12 strains carrying different peptidase enzymes, as a means of further analysing the effect of different *E. coli* K12 peptidases on growth properties and to confirm the peptide hydrolysing properties of each enzyme. Lysates of the P1 phage can transduce any marker on the *E. coli* K12 chromasome at a frequency of 10^{-4}-10^{-5} per infected cell by a process in which phage DNA is replaced by bacterial DNA (Miller, J.H., 1972), and so, combining this method of gene transfer with an appropriate strain selection procedure for resulting peptidase carrying recombinants, allows for simple construction of appropriate strains.

After initial studies revealed that previously isolated *E. coli* K12 strains, deficient in a number of broad specificity peptidases, retained the ability to utilise L-α-aspartyl peptides for growth, a number of different strategies were used in an effort to obtain mutant *E. coli* K12 strains defective in the ability to use these peptides as sources of required amino acids. Such strains would prove useful in investigations of the roles of particular peptidases in aspartyl peptide degradation *in vivo*, and their individual enzyme specificities, as well as facilitating later isolation of aspartyl peptidases and studies on their *in vivo* regulation. Using a method based on the observed sensitivity of *E. coli* K12 strains to the

amino acid valine (Miller & Schwartz, 1978), it was reasoned that if an appropriate strain was grown in the presence of the dipeptide Asp-Val, mutant cells unable to hydrolyse the dipeptide would not be exposed to the toxic effects of the constituent valine. This would provide a means for positively selecting aspartyl-peptidase deficient strains as under appropriate conditions parent strains (non-mutants) would be killed off by toxic levels of valine, and only spontaneous valine resistant mutants or mutants deficient in Asp-Val hydrolysis, would grow. In some cases this method of positive selection was preceded by mutagenesis of parent cultures with N-methyl-N'-nitrosoguanidine (NTG) or phage MudI (Ap,lac). Both mutagenic procedures cause random mutations around the E. coli K12 chromosome, in the case of NTG mediated via chemical damage of the DNA (Miller, 1972) and with phage MudI (Ap,lac) mediated by phage integration in the chromosome (Silharvy et al., 1987). Phage MudI (Ap,lac) mutagenesis of E. coli K12 strains was also used in experiments aimed at direct selection of aspartyl-peptide non-utilising mutants, and in combination with a colorimetric microassay aimed at detecting strains possessing lower levels of aspartyl peptidase activity (Carter & Miller, 1984).

Qualitative growth tests with all microorganisms and the genetic techniques employed for manipulations with E. coli K12 were carried out hand in hand with qualitative

peptidase assays. Polyacrylamide gel electrophoresis on non-denaturing gels was used for the separation of peptidase enzymes in microbial preparations (mostly crude cell extracts) enabling detection of peptidases with differing mobilities and substrate specificities when combined with a colorimetric assay (Lewis & Harris, 1967; Miller & Mackinnon, 1974). This assay depends on the following sequence of reactions

$$\text{peptide} + H_2O \xrightarrow{\text{Peptidase}} \text{L-amino acids} \qquad (1)$$

$$\text{L-amino acid} + O_2 \xrightarrow[\text{oxidase}]{\text{L-amino acid}} \text{keto acid} + NH_3 + H_2O_2 \qquad (2)$$

$$H_2O_2 + \text{O-dianisidine} \xrightarrow{\text{Peroxidase}} \text{oxidised dianisidine} \qquad (3)$$

The free amino acid liberated by peptide hydrolysis (1) is subjected to oxidative deamination by L-amino acid oxidase (2). The complete reaction results in the appearance of a dark brown zone at the site of peptidase activity (3). The assay works well for peptides containing leucine, isoleucine, phenylalanine, tyrosine, methionine and tryptophan; is weakly active with arginine, valine and histidine, and inactive with aspartate, asparagine, cysteine, glutamine, glycine, alanine, serine, lysine and proline. The inactivity of the assay with respect to aspartate put some limitations on the use of the assay in this work, as for example hydrolysis of peptides such as Asp-Glu, Asp-Gly would not be detected using this system.

3.2 Results

3.2.1 Preliminary Experiments

3.2.1.1 Confirmation of Peptide Purity

All peptides used in this work were supplied by the manufacturers at a purity of greater than 90% (w/w). Growth tests carried out on the peptidase deficient E. coli K12 leucine auxotroph, strain CM89, using a single batch of the dipeptide Leu-Gly gave unexpected and anomalous results. Strain CM89 was reported to be unable to use the dipeptide as a source of required leucine (Miller & Schwartz 1978) but in the experiments with this particular batch of Leu-Gly, growth of strain CM89 was observed. Analytical testing by thin layer chromatography combined with ninhydrin detection, showed that this particular batch of dipeptide was chromatographically heterogeneous, containing significant amounts of leucine (>10% w/w). Repetition of the growth tests using chromatographically homogeneous Leu-Gly in another batch from the same suppliers, showed no growth of strain CM89 on the pure dipeptide confirming that leucine impurity in the dipeptide sample had caused the observed CM89 growth.

All peptides subsequently used were subject to purity tests using TLC combined with ninhydrin detection. Mobilities for each peptide and constituent amino acids in 2 different solvent systems are shown in Table 2. Each peptide was separable into its component amino acids with

one of the solvent systems. Although it was recognised that amino acids varied in their sensitivity to ninhydrin detection, the amino acid aspartate was used as a control for estimating the limit of detection for this system. Aspartate was detectable on TLC plates after a load of 133 ng (10 µl of 0.01 mM Asp) but no amino acid spot was visible on loading 13.3 ng (10 µl of 0.001 mM Asp). The standard load of peptide in purity determination experiments was 10 µl of 0.01M peptide representing more than 20,000 ng peptide. Chromatographic homogeneiity therefore represented an estimated level of greater than 99% peptide (w/w), a level of amino acid impurity too low to support significant microbial growth in the experiments described in this work. Only peptides shown to be chromatographically homogeneous were used in further quantitative and qualitative growth tests.

3.2.1.2 Identification of Microorganisms

The identities of an E. coli K12 strain PN31, isolated in this chapter, and of the bacterial strain Acinetobacter catcoaceticus var anitratus were confirmed by a combination of microscopic analysis and API tests. The slow growth rate of PN31 and its genetic antecedents, combined with what at the time was considered to be the unexpected isolation of this aspartate auxotroph led to some doubts about the strain identity. The use of the A. calcoaceticus strain in later work for the isolation of an aspartyl-β-

Table 2 Amino Acid and Peptide Mobilities (R/f) after Thin Layer Chromatography on Cellulose MN300 Plates

Mobility (R/f)[a]	Solvent System[b]	
Amino acid/Peptide	(1)	(2)
Aspartate	0.05	0.24
Glutamate	0.05	0.32
Glycine	0.08	0.20
Valine	0.25	0.53
Lysine	0.07	0.17
Leucine	0.38	0.66
Phenylalanine	0.38	0.60
PheOMe	1.00	0.91
Tyrosine	0.27	0.48
Alanine	0.12	0.35
Proline	0.15	0.42
Asp-Leu	0.18	0.62
Asp-Gly	0.15	0.20
Asp-Glu	0.3	0.35
Asp-Lys	0.02	-
Asp-PheOMe	0.70	0.87
Asp-Phe	0.18	0.50
Asp-Ala	0.10	0.3
Asp-Tyr	0.32	0.28
Asp-Val	0.06	0.66
Asp-Pro	0.12	0.42
Phe-Asp	0.10	0.52

Table 2 (continued)

Mobility (R/f)[a]	Solvent System[b]	
Amino acid/Peptide	(1)	(2)
Glu-Phe	0.2	0.6
Leu-Gly	0.61	0.66
Leu-Leu	0.65	0.89
Leu-Pro	0.43	0.70
Leu-Arg	0.7	0.65
Leu-Ala-NH_2	0.76	0.63
Leu-Gly-Gly	0.29	0.56
Pro-Leu	-	0.74
Phe-Leu	0.63	0.89
Phe-Phe	0.69	0.88
Phe-Gly	0.37	0.63
Phe-Gly-Gly	0.29	0.58

[a] Mobilities (R/f) were determined as the distance moved by the amino acid/peptide divided by the distance moved by the solvent front

[b] Solvent (1) consisted of 2-methyl-2-butanol-butanone-propanone-methanol-water - 0.88 ammonia (50:20:10:5:15:5)
Solvent (2) consisted of butanol-acetic acid-water (4:1:1)

napthylamide hydrolysing enzyme meant that it was important for the identity of this strain to be confirmed.

Isolates of these species were shown to be Gram-negative. E. coli K12 cells were rod-shaped but contrary to the species description (Bergey, 1984) cells of A. calcoaceticus were coccoid in shape when Gram-stained cells were viewed through the light microscope rather than rod-shaped. API tests confirmed the strains as the designated species with 99% certainty for both microrganisms.

3.2.2 Studies on Aspartyl-Peptide Utilisation by E. coli K12

3.2.2.1 Peptide Utilisation Profiles of Peptidase Carrying and Peptidase Deficient Strains

Qualitative growth tests using different peptides as sources of required amino acids were carried out on previously isolated E. coli K12 strains to confirm peptide utilisation properties ascribed to these strains in other studies (Miller & Schwartz, 1978). Growth tests were performed by both streak plate and soft agar layer methods. Results of these growth tests for the peptidase carrying strains AB1157 and CM17, as well as the peptidase deficient strain CM89 (lacking peptidases D,N,A,B and Q) are shown in Table 3. Strain AB1157, presumed to carry a full complement of peptidases, was able to grow on all leucyl and prolyl peptides supplied as sources of the leucine and proline

Table 3. Amino Acid and Peptide Utilisation Profiles of Peptidase Carrying and Peptidase Deficient Strains of *E. coli* K12

Peptides/Amino acids	Utilisation by Strain		
	AB1157 (———)[a]	CM17 (D/NABQ)[b]	CM89 (DNABQ/-)
As Leu Source			
Leu	+	+	+
Leu-Gly	+	+	-
Leu-Pro	+	+	-
Leu-Ala-NH_2	+	+	-
Leu-Gly-Gly	+	+	-
Pro-Leu	+	+	-
Phe-Leu	+	+	-
Leu-Leu	+	+	-
Asp-Leu	+	+	+
As Pro Source			
Pro	+	+	+
Leu-Pro	+	+	-
Pro-Leu	+	+	-

[a] Peptidases present not known

[b] Parentheses indicate peptidases absent/peptidases present.

required by this strain. The 2 CM type strains (Miller & Schwartz, 1978) showed substantially different patterns of peptide utilisation. Whereas the peptidase carrying CM17 was able to use all peptides tested as sources of required leucine and proline, the only peptide capable of supplying leucine for strain CM89, was the aspartyl peptide Asp-Leu. No tested prolyl peptide would provide a source of proline for strain CM89.

The leucyl-peptide utilisation properties of strains CM17 and CM89 have been studied and their peptidase genotypes and phenotypes established (Miller & Schwartz, 1978). These workers found that strain CM17 carried 4 broad specificity peptidases enabling use of a broad range of peptide substrates for growth, while strain CM89 is deficient in these peptidases and has a limited range of peptide substrate utilisation.

According to this earlier study, the dipeptide Asp-Leu would not serve as a source of leucine for strain CM89, contrary to the result shown in Table 3; all other peptide utilisation tests correspond with the earlier study. The observation that strain CM89 can use the dipeptide Asp-Leu as a source of leucine for growth suggested the presence of a previously unreported Asp-Leu hydrolysing activity in this strain. The growth test data (Table 3) suggested that this

peptidase activity would not hydrolyse a variety of leucyl and prolyl peptides.

3.2.2.2 Polyacrylamide Gel Electrophoresis and Peptidase Activity Staining of Crude Cell Extracts from Peptidase Carrying and Peptidase Deficient strains

The data from initial growth tests suggested the presence of a previously unreported Asp-Leu hydrolysing enzyme in the E.coli K12 strain CM17 and its peptidase deficient derivative CM89. Peptidase profiles of these strains were investigated to ascertain the presence or absence of this novel Asp-Leu hydrolytic activity and to confirm previously ascribed peptidase profiles (Miller & Schwartz, 1978). Sonicated crude cell extracts were prepared from strains CM17 and CM89, and after separation of the extract proteins by polyacrylamide gel electrophoresis (PAGE) on 7.5% non-denaturing gels, the resultant gels were incubated with staining mixture containing appropriate peptide substrates. Gels were stained for peptidase activity towards the 2 leucyl dipeptides Leu-Gly and Leu-Pro and the 2 aspartyl peptides Asp-Leu and Asp-PheOMe (Fig. 1).

The PAGE separated extract from the peptidase carrying strain CM17 gave 3 bands of Leu-Gly hydrolysing activity at mobilities of 0.02, 0.3 and 0.6 (Fig. 1). These activity bands correspond in mobility with the 3 Leu-Gly hydrolysing activities previously described in this strain, designated

peptidases A, B and N (mobilities reported as 0, 0.3 and 0.6 respectively). The PAGE separated cell extract from the peptidase deficient strain CM89 gave no activity bands with Leu-Gly as assay substrate on the stained gel confirming the absence in this strain of peptidases capable of hydrolysing this substrate. Using Leu-Pro as assay substrate, the strain CM17 extract produced a single band of activity, mobility 0.45, whereas the CM89 extract gave no bands of activity (Fig. 1). The strain CM17 Leu-Pro hydrolytic activity corresponds in mobility with the previously described Leu-Pro hydrolysing peptidase Q. No Leu-Pro activity band corresponding in mobility with the previously described peptidase P was observed in these assays.

Incubation of the separated crude extracts with either Asp-PheOMe or Asp-Leu and gel staining for peptidase activity produced 2 bands of activity, mobilities 0.3 and 0.5, with the peptidase carrying CM17 extract, and a single band of hydrolytic activity mobility 0.5 with the cell extract from the peptidase deficient CM89. The observation of the aspartyl peptide hydrolysing band at mobility 0.5 had not been previously reported, and this confirmed the indication from growth experiments that an additional previously undescribed peptidase was present in these K12 strains. This aspartyl peptide hydrolysing activity was not observed in gels where either Leu-Gly or Leu-Pro were assay substrates, suggesting that the novel peptidase could be

Figure 1. Peptidase Activity Profiles Of Crude Cell Extracts From E.coli CM17 And CM89 After Seperation And Staining On Polyacrylamide Gels

Strain		Substrate
E.coli CM17	Gel 1	Leu-Gly
	Gel 2	Asp-PheOMe
	Gel 3	Asp-Leu
	Gel 4	Leu-Pro
E.coli CM89	Gel 5	Leu-Gly
	Gel 6	Asp-PheOMe
	Gel 7	Asp-Leu
	Gel 8	Leu-Pro

key

Activity Band

Bromophenol Blue

ACTIVITY BAND 0·3 (PEPTIDASE B)

ACTIVITY BAND 0·5 (PEPTIDASE E)

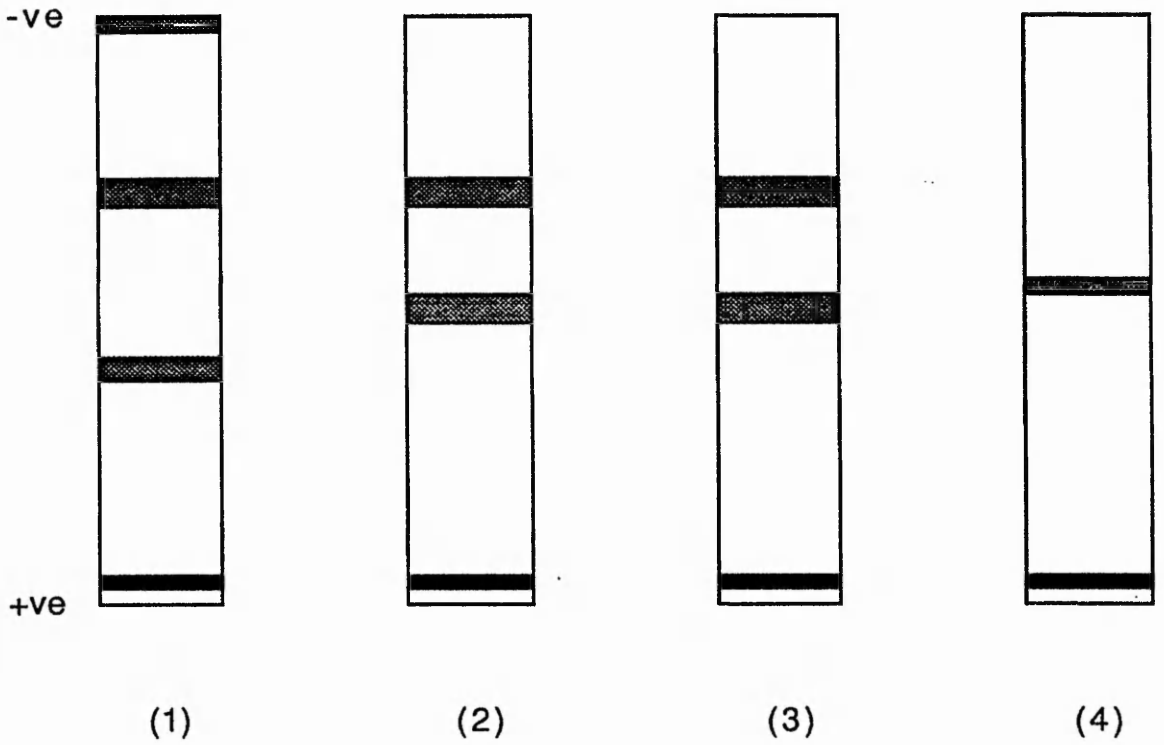
PHOTO GEL (3)

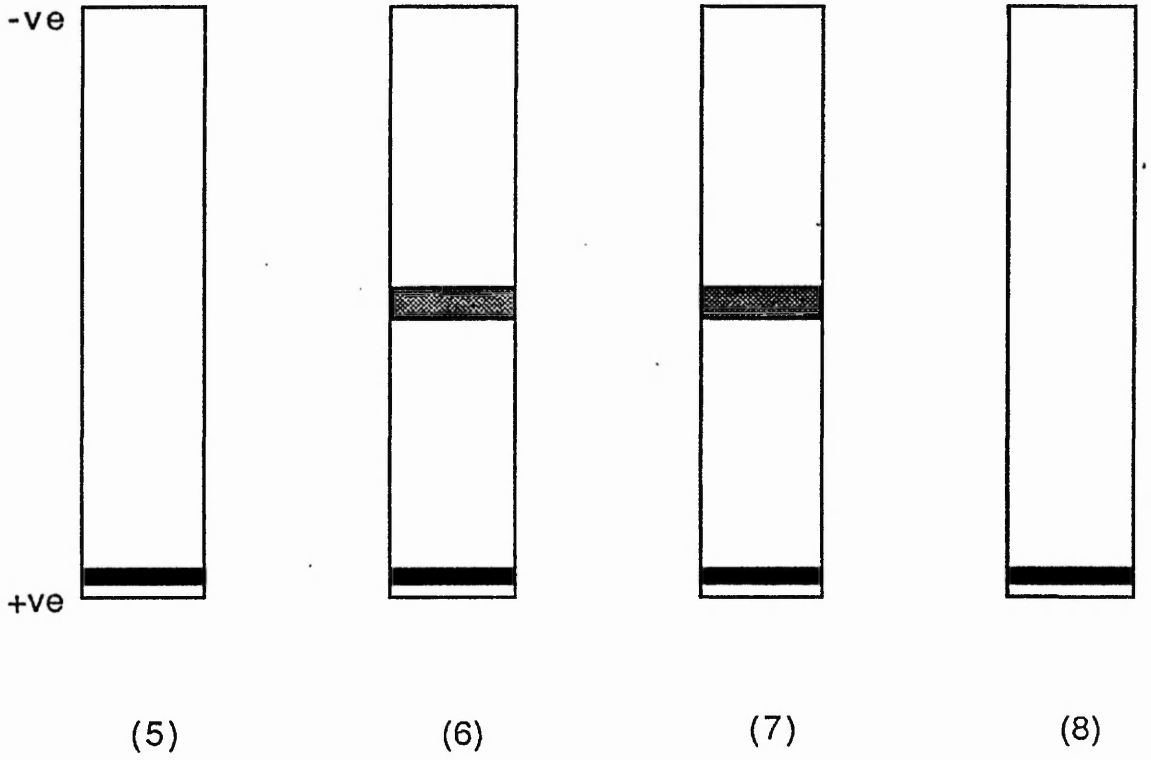
E. coli CM17 Asp-Leu Substrate

ACTIVITY BAND 0·5 (PEPTIDASE E)

PHOTO GEL (7)

E. coli CM89 Asp-Leu Substrate


-ve
+ve
(1)
(2)
(3)
(4)


-ve
+ve
(5)
(6)
(7)
(8)

specific for aspartyl peptides. Such an aspartyl peptide specific enzyme, designated peptidase E, has been described in Salmonella typhimurium (Carter & Miller, 1984). The mobilities of the S. typhimurium peptidase E activity and the K12 aspartyl peptide cleaving activity observed in strains CM17 and CM89, differ wih the S. typhimurium activity reported as running at mobility 0.7 on 7.5% non-denaturing gels. The aspartyl peptidase activity observed at mobility 0.3 corresponds to the K12 peptidase B and this activity appears to hydrolyse both the dipeptide Leu-Gly as well as Asp-PheOMe and Asp-Leu. This peptidase B activity is shown to be absent in strain CM89 as no Leu-Gly hydrolysing activity is observed after peptidase activity staining, and the equivalent aspartyl peptide hydrolysing activity is also absent from these gels providing additional evidence that K12 peptidase B can hydrolyse aspartyl peptides. The gel data presented here also suggests that peptidases A and N have no activity towards L-α-aspartyl peptides as no activity bands corresponding to these peptidases were observed with either Asp-Leu or Asp-PheOMe as assay substrates. No activity bands were observed when aspartyl-β-napthylamide was used as gel assay substrate.

Pre-incubation of gels for 30 minutes in 1mM $MnCl_2$ produced no additional bands of peptidase activity although stimulation of the peptidase B activity, observed as a broadened band on the $MnCl_2$ incubated gels, appeared to

occur with both Leu-Gly, Asp-Leu and Asp-PheOMe as assay substrates. Pre-incubation in 1mM $CoCl_2$ produced no additional activity bands with either substrate, in contrast to a previous report of a Co^{2+} stimulated Leu-Gly hydrolysing activity mobility 0.28, designated aminopeptidase L observed, in crude extracts from both CM17 and CM89 (Miller & Schwartz, 1978).

3.2.2.3 Isolation of Streptomycin Resistant Derivatives of Peptidase Carrying and Peptidase Deficient *E. coli* K12 strains

The construction using genetic techniques of derivative of peptidase carrying and peptidase deficient *E. coli* K12 strains with an auxotrophic requirement for the amino acid phenylalanine would facilitate studies of aspartame (Asp-PheOMe) utilisation in this organism. The isolation of streptomycin resistant derivatives of both strains CM17 and CM89 was a prerequisite for subsequent conjugation experiments aimed at the construction of phenylalanine auxotrophic derivatives of these strains. Plating out of 0.1 ml aliquots from concentrated overnight nutrient broth cultures on nutrient agar plates containing 100 µg/ml streptomycin followed by incubation of plates at 37°C, generated approximately 10 putative streptomycin resistant colonies per plate, for both strains CM17 and CM89. Restreaking 10 colonies from each experiment on further streptomycin containing nutrient agar plates resulted in

growth after incubation of all colonies tested confirming streptomycin resistance. 3 colonies from each experiment were stored on nutrient agar slants at 4°C, and these strains were designated for CM17 resistant mutants PN1-3 and for CM89 streptomycin resistant mutants PN4-6. Amino acid auxotrophic markers and Leu-Gly utilisation properties were tested for these strains and each streptomycin resistant strain retained the parental phenotype.

3.2.2.4 Construction of Derivatives of Peptidase Carrying and Peptidase Deficient *E. coli* K12 Strains with an Auxotrophic Requirement for Phenylalanine

Phenylalanine amino acid auxotrophs derived from the streptomycin resistant peptidase carrying and peptidase deficient strains PN1 and PN4 respectively were constructed in order to broaden the range of peptide utilisation tests which could be carried out. The *E. coli* K12 Hfr strain KA197 carries a phenylalanine auxotrophic marker mapping at 56.8 minutes on the *E. coli* K12 genome (Bachmann, 1983), and this strain transfers genes in an anti-clockwise direction from an origin of transfer mapping at 63 minutes on mating with a recipient strain. One of the early markers transferred during mating with this Hfr strain is a thy^+, thymine utilisation marker mapping at around 61 minutes (Bachmann, 1983). Recombinant colonies from mating experiments between Hfr KA197 and the F^- thy^- strains PN1 and PN4 could therefore be selected on appropriate M9

minimal medium plates supplemented with phenylalanine and streptomycin but lacking thymine. Parental strains would not grow on this medium. Conjugational crosses were therefore carried out between these strains with sampling of mating mixtures after 10 and 20 minutes (Table 4). Recombinant colonies selected as thy^+ and $strp^r$ were tested for phenylalanine auxotrophy by replica plating onto phenylalanine containing and phenylalanine deficient minimal plates. Phenylalanine auxotrophic recombinants derived from the peptidase deficient strain PN4, were further tested to check that no broad specificity peptidase was transferred during conjugation by replica plating onto minimal plates where the dipeptide Leu-Gly was the sole source of required leucine. 90 out of 95 recombinant PN4 derived phenylalanine auxotrophs had become $Leu\text{-}Gly^+$ after conjugation, indicating transfer of a Leu-Gly hydrolysing peptidase from strain KA197. The gene encoding this peptidase appeared to be closely associated with the Phe^- marker however at this stage the identity of the transferred peptidase was not further investigated.

3 phenylalanine auxotrophic colonies derived from the peptidase carrying PN1, 3 derived from strain PN4 devoid in Leu-Gly hydrolysing activity and 3 derived from PN4 but carrying Leu-Gly hydrolysing activity, and were designated PN7-9, PN10-12 and PN13-15 respectively and these were stored at 4°C on nutrient agar slants.

Table 4 Analysis of Recombinant Colonies Isolated in *E. coli* K12 Conjugational Crosses

Donor Strain	Recipient Strain	Mating Time (mins)	Selection	No. of Recombinants Tested	No. of Recombinants			
					$Phe^+Leu\text{-}Gly^+$	$Phe^+Leu\text{-}Gly^-$	$Phe^-Leu\text{-}Gly^+$	$Phe^-Leu\text{-}Gly^-$
KA 197	PN1	10	thy^+strp^r	30	30	0	0	0
		20	thy^+strp^r	150	40	0	110	0
KA 197	PN4	10	thy^+strp^r	30	8	21	1	0
		20	thy^+strp^r	150	53	2	90	5

3.2.2.5 Introduction of Wild Type *pep* Alleles into the Peptidase-Deficient strain PN10 by P1 Transduction

Prior to analysis of the aspartyl and phenylalanyl peptide utilisation properties of the newly isolated phenylalanine auxotrophs, derivative strains were constructed, each carrying a single broad specificity peptidase. This would enable study of the effect of the individual peptidase enzymes on the peptide utilisation properties of the recipient strain giving some information on peptidase specificity, as well as providing confirmatory data on the role of each peptidase in aspartyl peptide degradation. It would also provide an opportunity to determine whether the broad specificity dipeptidase, peptidase D (Miller & Schwartz, 1978) would degrade aspartyl peptides, as this enzyme had not been studied in previous experiments.

Using strain CM17, carrying 4 wild type broad specificity peptidase alleles, as a donor and PN10, a strain deficient in these same 4 peptidase alleles, as a recipient, P1 transductional crosses were carried out. Recombinants able to use Leu-Gly as a leucine source were selected in one cross and Leu-Pro utilising recombinants in another. These were purified by single colony isolation and tested for the utilisation of leucyl-peptides and the ability to hydrolyse leucyl-β-napthylamide (Table 5). Comparison of the growth properties of recombinant strains on leucyl peptides, with

Table 5. Leucyl-Peptide Utilisation profiles of *E. coli* K12 Recombinants Generated by P1 Transduction

Recombinant Strain	LG	LGG	LA	LP	LNA	Genotype
PN16	+	+	+	-	-	pep A^+
PN19	+	+	-	-	-	pep B^+
PN22	+	+	+	-	+	pep N^+
PN25	+	-	-	+	-	pep Q^+
PN28	+	-	-	ND	-	pep D^+

Recombinants derived from transductional crosses CM17 x PN4; SA173 x PN4
Abbreviations LG, Leu-Gly; LGG, Leu-Gly-Gly; LA, Leu-Arg; LP, Leu-Pro; LNA, Leucyl-β-napthylamide. ND = not done (PN28 is a proline prototroph and growth on LP would therefore not necessarily indicate utilisation of this dipeptide).

data reported previously (Miller & Schwartz, 1978) showed that strains each carrying one of the wild-type pep alleles had been isolated. 3 colonies of each class of recombinant were stored on nutrient agar slants at 4°C and these strains carrying the functional pep alleles pep A, pep B, pep N, pep Q designated PN16-18, PN19-21, PN22-24 and PN25-27 respectively. A further transductional cross was carried out using E. coli K12 strain SA173 as donor and PN10 as recipients. Pro^+ recombinants were selected and these had all acquired the ability to use Leu-Gly as a leucine source. These strains were considered to be pep D^+ and 3 pep D^+ colonies were selected for storage and designated strains PN28-30.

Peptidase phenotypes of each recombinant strain were confirmed by qualitative peptidase assays after separation of crude extracts by polyacrylamide gel electrophoresis. Strains ascribed a particular peptidase phenotype showed the presence of the appropriate peptidase as judged by activity band mobility, on polyacrylamide gels (Fig. 2).

Additional bands of Leu-Gly hydrolysing activity were observed at mobilities 0.02, 0.3, 0.48 and 0.6 for the different strains carrying each of the peptidases A, B, D and N respectively. A single band of Leu-Pro hydrolysing activity, mobility 0.45 was observed in the pep Q^+ strain.

Figure 2. Leucyl-Glycine Hydrolysing Activity Profiles Of Recombinant E.coli K12 Strains Generated By P1 Transduction

Gel 1 PN10 (peptidase deficient parent)

Gel 2 PN16 (peptidase A activity band mobility 0.02)

Gel 3 PN19 (peptidase B " " " 0.3)

Gel 4 PN22 (peptidase N " " " 0.6)

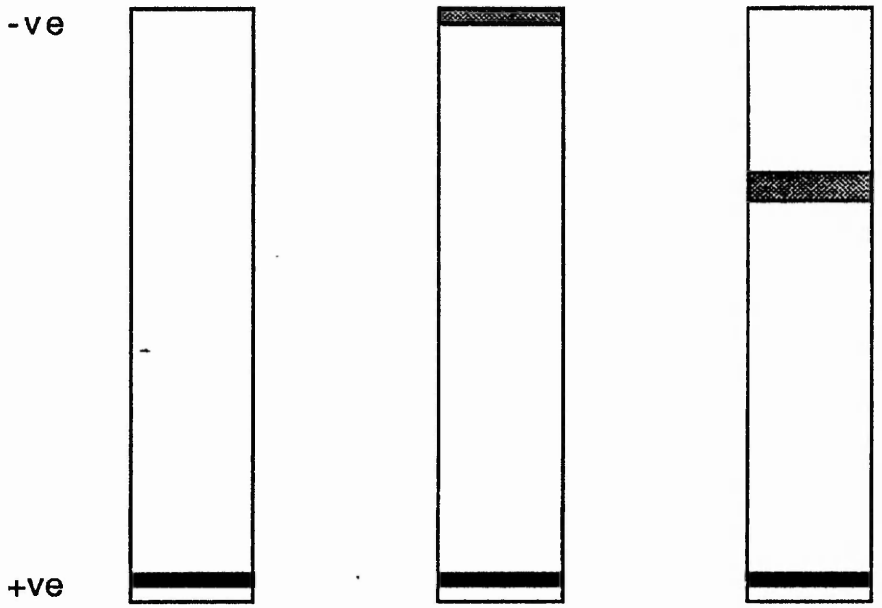
Gel 5 PN25 (peptidase Q " " " 0.45)

Gel 6 PN28 (peptidase D " " " 0.48)

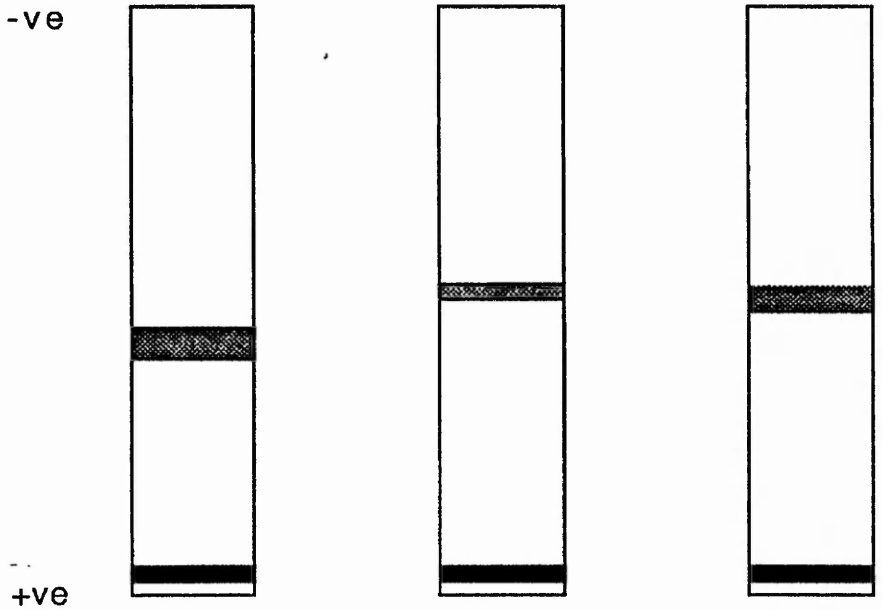
key

Activity Band

Bromophenol Blue



(1) (2) (3)



(4) (5) (6)

3.2.2.6 Peptide Utilisation Properties and Peptidase Profiles of Peptidase Carrying, Peptidase Deficient and Transductant Phenylalanine Auxotrophs

Qualitative growth tests were carried out using phenylalanine containing peptides as sole source of required phenylalanine for the peptidase carrying strain PN7, the peptidase deficient PN10, and the PN10 transductant derivatives PN16, PN19, PN22, PN25 and PN28, each carrying a single wild-type pep allele (Table 6). The aspartyl peptides, Asp-PheOMe (aspartame), Asp-Phe and Phe-Asp served as phenylalanine sources for all strains tested, including the peptidase deficient PN10, confirming the likelihood of novel aspartyl peptidases in peptidase deficient strains. A range of other phenylalanyl peptides were also found to serve as phenylalanine sources for each of the strains tested. The multiple peptidase deficient strain PN10 was able to use all phenylalanyl peptides except for Phe-Leu as growth substrates. This growth test data suggested the presence in these strains of additional peptidase activities responsible for phenylalanyl peptide hydrolysis other than the previously described peptidases A, B, D, Q and N. The broad specificity peptidases A, B, D and N appear to hydrolyse the dipeptide Phe-Leu, as transductant derivatives of the peptidase deficient PN10 each carrying one of these alleles, grew on the peptide as a source of phenylalanine.

Table 6. Patterns of Phenylalanyl Peptide Utilisation in Parent and Recombinant Strains of *E. coli* K12

Amino acid/Peptides	Utilisation by strain						
	PN7 (D/NABQ)[a]	PN10 (DNABQ/-)	PN16 (DABQ/A)	PN19 (DNBQ/B)	PN22 (DANQ/N)	PN25 (DANB/Q)	PN28 (ANBQ/D)
Phenylalanine source:							
Phe	+	+	+	+	+	+	+
PheOMe	+	+	+	+	+	+	+
Phe-Gly-Gly	+	+	+	+	+	+	+
Phe-Leu	+	-	+	+	+	-	+
Phe-Phe	+	+	+	+	+	+	+
Phe-Asp	+	+	+	+	+	+	+
Phe-Gly	+	+	+	+	+	+	+
Asp-Phe	+	+	+	+	+	+	+
Asp-PheOMe	+	+	+	+	+	+	+

[a] Parentheses indicate peptidases absent/peptidases present.

PAGE analysis of peptidase profiles in crude cell extracts (Fig. 3) revealed that the aspartyl-peptide hydrolysing activity observed at mobility 0.5 on gels from peptidase-deficient strain CM89 extracts, was also present in the peptidase-deficient phenylalanine auxotroph PN10. This activity was observed on gels stained for Asp-Phe, Asp-PheOMe and Asp-Leu hydrolysis but no activity band at 0.5 was observed with the C-terminal aspartyl peptide Phe-Asp as assay substrate. Moreover no bands of Phe-Asp hydrolysing activity were observed at all in cell extracts from strain PN10 or any of the other phenylalanine auxotrophic strains indicating that either the peptidase activity assay was not capable of detecting this peptidase activity or that Phe-Asp hydrolysing activity had been lost prior to assay. Peptidases A, B, D, Q and N gave no activity bands with Phe-Asp as assay substrate. The transductant derivative of PN10, carrying the wild-type pepB allele, strain PN19, showed a band of aspartyl-peptide hydrolysing activity at mobility 0.3 with either Asp-Leu, Asp-PheOMe or Asp-Phe as substrate, in addition to the 0.5 mobility band of the parent strain, confirming that peptidase B can hydrolyse aspartyl peptides. Transductant strains carrying wild-type pepA, pepD, pepN and pepQ alleles showed no bands of aspartyl peptidase activity additional to the parent strain, indicating that the respective peptidases do not hydrolyse aspartyl peptides.

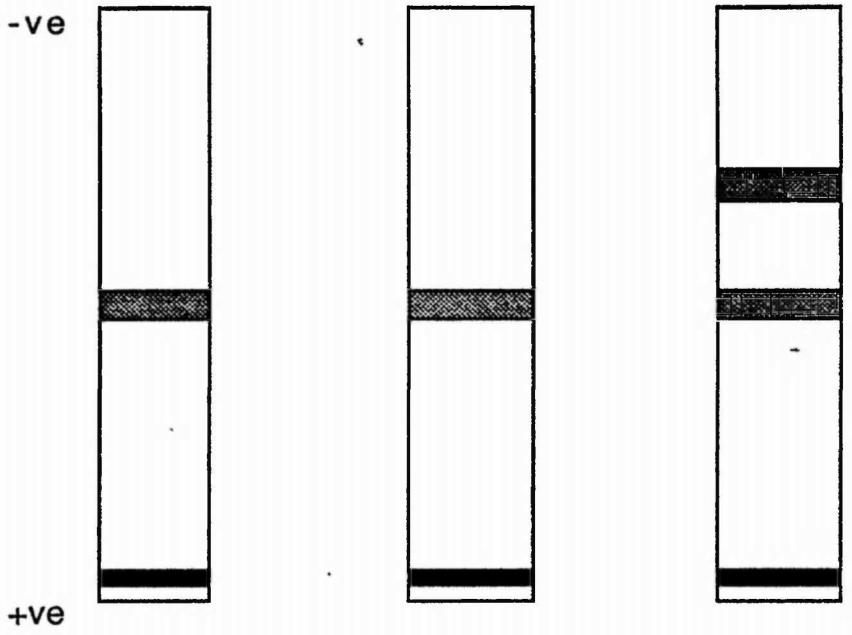
Figure 3. Aspartame / Asp-Leu Hydrolysing Activity Profiles Of Recombinant E.coli K12 Strains Generated By P1 Transduction

Gel 1	PN10	(single activity band mobility 0.5)
Gel 2	PN16	(" " " " ")
Gel 3	PN19	(activity bands mobility 0.5, and 0.3)
Gel 4	PN22	(single activity band mobility 0.5)
Gel 5	PN25	(" " " " ")
Gel 6	PN28	(" " " " ")

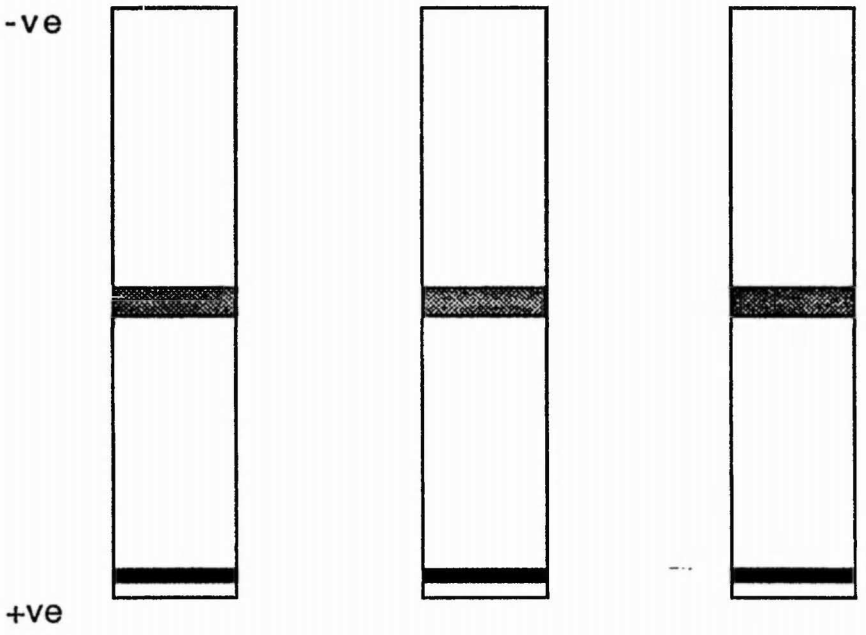
key

Activity Band

Bromophenol Blue



(1) (2) (3)



(4) (5) (6)

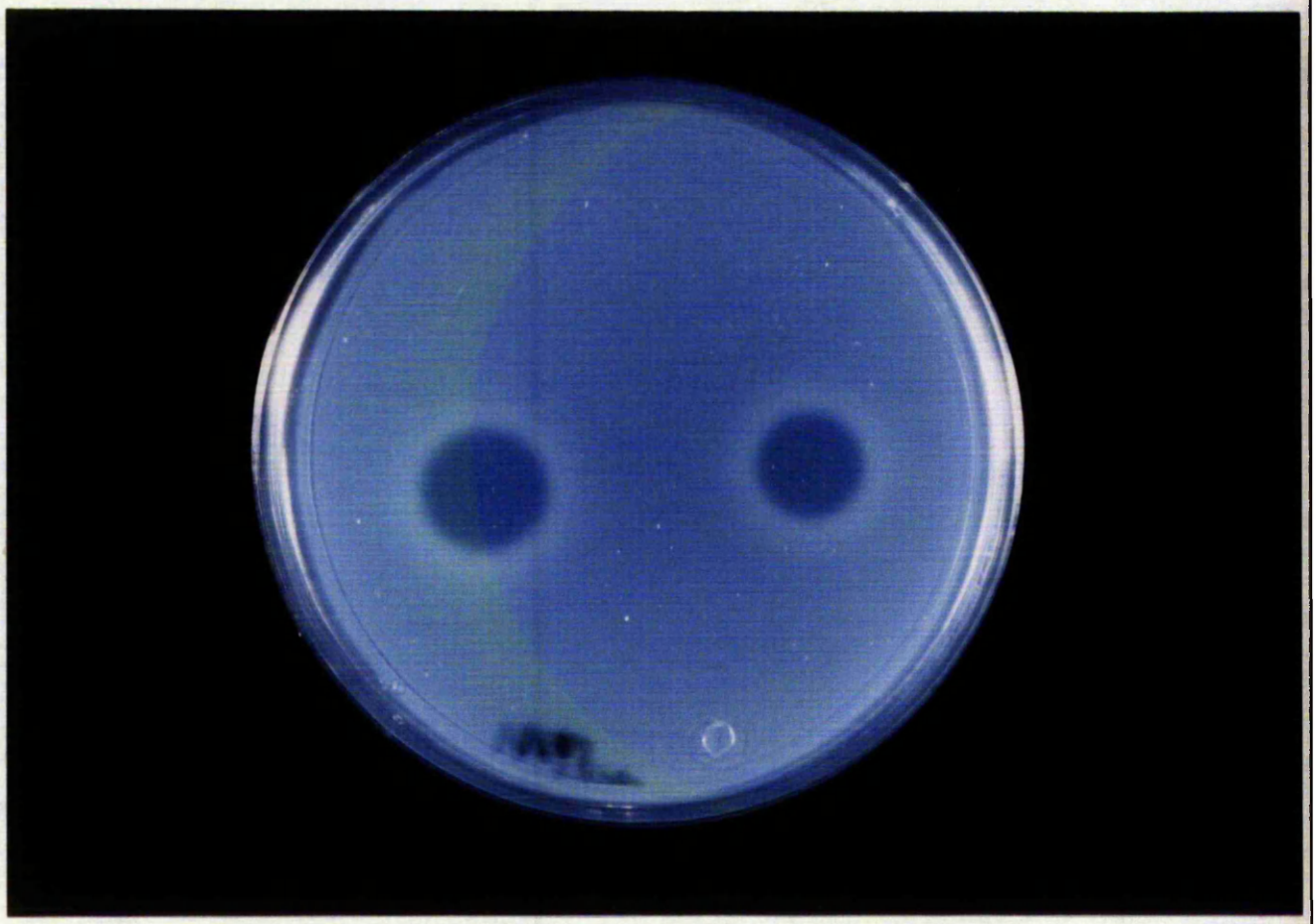
Pre-incubation of gels in 1mM $MnCl_2$ or $CoCl_2$ prior to peptidase assay did not stimulate the production of any additional bands of peptidase activity towards aspartyl peptides, although the presence of Mn^{2+} appeared to stimulate peptidase B activity as evidenced by a broadening of the respective activity band.

PAGE separated cell extracts were also stained for peptidase activities with phenylalanyl peptides as assay substrates. With the tripeptide Phe-Gly-Gly as substrate, extracts from all strains produced a single band of peptidase activity mobility 0.45, possibly representing a tripeptidase, designated peptidase T, previously described (Simmonds et al., 1976; Strauch et al., 1983). Strains PN19 and PN22 carrying peptidase B and N respectively gave additional activity bands mobility 0.3 and 0.6 respectively with Phe-Gly-Gly as assay substrate. With the assay substrate Phe-Gly, only the Leu-Gly hydrolysing dipeptidase D (mobility 0.48) appeared to show activity but the peptidase deficient strain PN10 although able to use this peptide as a source of required phenylalanine, showed no additional Phe-Gly hydrolysing activities providing another example of a peptidase activity not detected under the conditions used in this assay system. Pre-incubation of gels with 1mM $MnCl_2$ or $CoCl_2$ produced no additional activity bands.

3.2.2.7 Aspartyl Valine and Valine Sensitivity Tests

The initial strategy adopted for the isolation of E. coli K12 mutants defective in aspartyl peptide hydrolysing activity involved the isolation of Asp-Val resistant strains that were also valine sensitive. Such mutants might be deficient in aspartyl peptidase activity and these would be useful for genetic, physiological and biochemical studies of peptidase enzymes. The multiply peptidase-deficient strain PN10 was used in mutant isolation experiments as only a single aspartyl peptidase activity had been detected in this strain. The valine and aspartyl-valine sensitivity of strain PN10 was investigated before attempting to isolate the mutants. Spotting of 5mM Asp-Val and Val on minimal agar plates overlaid with soft agar containing overnight culture of strain PN10 gave rise to areas of growth inhibition around both dipeptide and amino acid (Fig. 4). The diameters of the zones of inhibition for peptide and amino acid were similar (12mm and 13mm respectively) indicating a similar degree of toxicity. It was concluded that strain PN10 was valine sensitive and that an aspartyl-valine hydrolysing activity was responsible for the release of the amino acid residue valine leading to a toxic Asp-Val effect. Spontaneous valine resistant mutants were also resistant to Asp-Val indicating that the dipeptide itself was not toxic.

Figure 4. Inhibition of Growth of the Multiply Peptidase-Deficient *E. coli* PN10 by Valine and Aspartyl Valine. (Clear areas represent zones of Inhibition: left hand side valine, right hand side Asp-Val)



Further tests were carried out to determine the critical concentration of Asp-Val and valine at which growth of strain PN10 would be suppressed on minimal agar plates. Abundant growth of the strain was observed on minimal plates containing 10^{-6}M and 10^{-5}M dipeptide and amino acid while only occasional spontaneous valine resistant colonies were observed on minimal plates containing 5 x 10^{-4}M or 10^{-4}M amino acid or peptide. The critical concentration for both Asp-Val and valine inhibition was 10^{-4}M and this concentration of amino acid and dipeptide was used in minimal plates in subsequent mutant isolation experiments.

3.2.2.8 Isolation and Analysis of Spontaneous Aspartyl-Valine Resistant Mutants Derived from the Peptidase Deficient Strain PN10

Concentrated PN10 overnight cultures were plated out on minimal medium supplemented with 10^{-4}M Asp-Val. Colonies appearing after 48 hrs incubation at 37°C were replica plated onto further minimal plates supplemented with either 10^{-4}M Asp-Val or 10^{-4}M valine. Of 2,000 putative Asp-Val resistant colonies isolated, 21 colonies grew on the Asp-Val plates but not the valine plates. These colonies were purified and tested for their ability to utilise the aspartyl peptides Asp-Leu, Asp-PheOMe and Asp-Phe as sources of required amino acids. All of these newly isolated Asp-Val resistant strains retained the parental ability to grow on these peptide substrates. However one isolate,

designated strain PN31, did not grow on control plates containing only amino acid growth requirements. Further growth tests showed that strain PN31 had an auxotrophic requirement for aspartate.

Cell extracts were prepared from each of the Asp-Val resistant strains and used for peptidase profile analysis after separation of proteins by polyacrylamide gel electrophoresis. Staining gels for activity towards Asp-Leu and Asp-PheOMe showed that all of these resistant strains retained the parental aspartyl peptide hydrolysing activity mobility 0.5. No evidence of a loss of aspartyl peptidase activity was therefore demonstrated using this assay in these Asp-Val resistant strains. The Asp-Val resistant phenotype could possibly be caused by a defect in uptake of the particular peptide or by the absence/reduced levels of an Asp-Val hydrolysing peptidase not detectable in gel assays. The amino acids valine and aspartate are poor substrates for the gel asay and Asp-Val degrading activities could not be detected using this system. The newly isolated aspartate auxotroph PN31 and the Asp-Val^{r} Val^{s} mutants, designated PN32-52, were stored on nutrient agar slants at 4°C.

3.2.2.9 Peptide Utilisation Properties of the Peptidase-Deficient Aspartate Auxotroph PN31 and its Transductant Derivatives: The Nature of Aspartate Auxotrophy

Preliminary attempts to isolate aspartyl-peptidase deficient *E. coli* K12 strains by selection for Asp-Val^r Val^s derivatives of strain PN10 had fortuitously resulted in the isolation of the aspartate auxotroph strain PN31. The aspartate requirement of PN31 would enable a wide range of aspartyl peptides to be tested as growth substrates in peptide utilisation experiments performed with this peptidase deficient strain.

The isolation of the aspartate auxotroph was unexpected as the literature seemed to suggest that a double mutant was required for *E. coli* K12 strains to show aspartate auxotrophy (Hermann 1980; Gelfand & Steinberg 1978), with mutations required in the *aspC* gene encoding aspartate transaminase, and in the *tyrB* gene encoding tyrosine transaminase. Such an aspartate auxotroph would have a simultaneous tyrosine growth requirement not exhibited by strain PN31. One possibility considered was that phenylalanine supplied in the medium is hydroxylated to tyrosine replacing the requirement for exogenous tyrosine. However no such process of phenylalanine hydroxylation has been described in *E. coli* K12 nor has any phenylalanine hydroxylase been reported in this organism. Alternatively

the absence of a metabolic precursor required for aspartate biosynthesis might preclude growth in the absence of exogenous aspartate. Various biosynthetic precursors of aspartate were included in minimal medium plates in place of aspartate and PN31 culture streaked out on these plates. Replacement of aspartate with oxaloacetate, pyruvate, phosphoenol pyruvate and α-ketoglutarate produced no growth with PN31.

P1 transductional crosses were carried out as in 3.2.2.5 using peptidase carrying strains CM17 and SA173 as donor strains but with the peptidase-deficient aspartate auxotroph PN31 was recipient culture. This would enable analysis of the effects of peptidase acquisition on aspartyl-peptide utilisation profiles. Transductants were selected as in 3.2.2.5, each carrying one of the peptidases A', B, D, Q and N and following confirmation of the peptidase phenotype by the gel stain assay, 3 colonies of each type were stored and designated PN53-55, PN56-58, PN59-61, PN62-64 and PN65-67 respectively.

Growth tests were then carried out on strain PN31 and its transductant derivatives using aspartyl peptides as a source of required aspartate (Table 7). All aspartyl peptides tested would support growth of the peptidase deficient PN31 except for Asp-Pro, and no degradation of Asp-β-napthylamide could be detected with this strain. The

Table 7. Patterns of Aspartyl Peptide Utilisation in Parent and Recombinant strains of E. coli K12

Amino acid/Peptides	Utilisation by strain					
	PN31 (DNABQ/-)[a]	PN53 (DNBQ/A)	PN56 (DNAQ/B)	PN59 (DABQ/N)	PN62 (DANB/Q)	PN65 (ANBQ/D)
Aspartate Source						
Asp	+	+	+	+	+	+
Asp-Phe	+	+	+	+	+	+
Asp-PheOMe	+	+	+	+	+	+
Asp-Leu	+	+	+	+	+	+
Asp-Val	+	+	+	+	+	+
Asp-Tyr	+	+	+	+	+	+
Asp-Pro	-	-	-	-	+	ND
Asp-Glu	+	+	+	+	+	+
Asp-Gly	+	+	+	+	+	+
Asp-Lys	+	+	+	+	+	+
Asp-β-napth	-	-	-	-	-	-
Asp-Ala	+	+	+	+	+	+
Phe-Asp	+	+	+	+	+	+
CBZ-Asp	+	+	+	+	+	+

a) Parentheses indicate peptidases absent/peptidases present
ND = Not Done

pepQ$^+$ transductant derivative of PN31, gained the ability to use Asp-Pro as a source of aspartate suggesting that this prolyl-peptide specific enzyme includes Asp-Pro within its range of substrates. The other peptidase carrying transductants showed the same patterns of aspartyl peptide utilisation as their parent PN31. No differences in the substrate specificities between the aspartyl peptide hydrolysing enzyme peptidase B and the aspartyl peptidase activity mobility 0.5 on activity gels, could be determined using these growth tests.

3.2.2.10 Mutagenesis of the Peptidase Deficient Aspartate Auxotroph PN31 using N-methyl-N'-nitrosoguanidine (NTG) or Phage Mud I (Ap,lac): Selection for Mutants Defective in Aspartyl Peptide Utilisation and Degradation

Cultures of strain PN31 mutagenised with either NTG or phage Mud I (Ap,lac) were plated out on minimal media supplemented with 10^{-4}M Asp-Val. Putative Asp-Val^r colonies were further tested to confirm this growth phenotype and determine valine sensitivity. None of the 1,000Asp-Val^r Val^s colonies isolated were defective in aspartyl peptide utilisation and of 20 isolates tested none proved defective in aspartyl peptidase activity as determined by gel assays.

The efficiency of the NTG mutagenesis procedure was confirmed by plating samples of mutagenised PN31 on minimal plates containing 10^{-4}m Val and comparing the resulting numbers of Val^r colonies with those obtained from non-mutagenised cells. A 3,000-fold increase in Val^r colonies was observed after NTG mutagesis.

As selection of Asp-Val^r mutants from strain PN31 had not resulted in the isolation of a mutant confirmed as defective in aspartyl-peptidase activity, or aspartyl peptide utilisation, it was decided to select directly for aspartyl-peptide non-utilizing strains. Strain PN31 was mutagenised with phage MudI (Ap,lac) and ampicillin resistant mutants isolated. 5,000 such ampicillin resistant colonies were tested for their ability to grow on aspartyl peptides as source of required amino acids. None of these colonies were defective in the ability to utilise either Asp-Leu, Asp-Glu, or Asp-PheOMe as sources of aspartate.

The difficulty in selecting aspartyl-peptide non-utilising mutants led to the suggestion that the peptidase deficient PN31 might carry several aspartyl-peptidase encoding genes, and several aspartyl peptidases with overlapping specificities, which were not detectable using the gel assay system. 4 broad specificity peptidases capable of hydrolysing leucyl peptides have been described in E. coli K12 (Miller & Schwartz 1978) and 4 aspartyl

peptide hydrolysing enzymes have been reported in Salmonella typhimurium (Carter & Miller, 1984).

As only a single band of aspartyl-peptidase activity had been observed in crude cell extracts of the peptidase-deficient PN31 on PAGE gel assays, it was decided to see if it would be possible to select for mutants lacking this particular peptidase activity. Mutants lacking this activity would be unable to generate the orange-brown colour observed on activity staining of gels. To simplify the selection procedure colorimetric microassays (Carter & Miller, 1984) based on the gel assay system were used. 2,000 ampicillin resistant colonies generated by MudI (Ap,lac) mutagenesis of strain PN31 were subjected to microassay in microtitre plates. None of these mutagenised colonies were defective in generation of the orange-brown colour and hence none were observed as defective in the aspartyl-peptide hydrolysing activity band observed on PN31 PAGE assay gels.

3.2.2.11 Peptidase Profiles from Concentrated Cell Extracts of Peptidase-Deficient Strains

The inability to isolate aspartyl-peptidase deficient mutants from peptidase-deficient strains suggested the possible existence of multiple aspartyl peptidases. Only a single aspartyl peptidase activity had been detected in these strains on gel staining of separated crude cell

extracts. In an effort to detect new activities, cell extracts of the peptidase deficient strains CM17, PN10 and PN31 were concentrated by ammonium sulphate fractionnation to give protein concentrations of around 25 mg/ml, and these fractions separated by PAGE then stained for aspartyl peptidase activity. No additional bands of Asp-Leu or Asp-PheOMe hydrolysing activity were observed on these gels. Pre-incubation of these gels in 1mM $CoCl_2$ or 1mM $MnCl_2$ produced no additional bands of aspartyl peptidase activity.

In S. typhimurium LT2 additional bands of aspartyl peptidase activity were reportedly observed by increasing amounts of cell extract loaded onto gels, combined with a longer (3-6 hrs) period of colour development on gels and an incubation temperature of 37°C (Carter & Miller, 1984). None of these altered conditions when applied to E. coli K12 extracts produced additional bands of aspartyl peptidase activity. A number of factors could account for the non-appearance of additional peptidase activities on gel staining of crude extracts. Such peptidases might be sensitive to the procedures used for the production of crude cell extracts, through sonication sensitivity, temperature sensitivity or instability in buffers. Alternatively these peptidases may not be active in the hostile environment of the gel assay system, or require co-factors not supplied in these assays. The peptidase M activity from S. typhimurium LT2 is not detectable on gel assay systems (Miller et al.,

1987). The inability to isolate mutants defective in the *E. coli* K12 aspartyl-peptidase, colour forming activity, mobility 0.5 on PAGE gels, using the colorimetric microassay indicates the presence of additional colour forming enzyme activities on microassay. It seems possible that additional aspartyl-peptidases are stable for the microassay enzyme isolation procedure but not to isolation by the sonication procedure. This phenomenon was not investigated further. Experimental work in the subsequent sections focuses on the properties of the aspartyl-peptide hydrolysing peptidase B and the novel aspartyl-peptidase activity mobility 0.5 on PAGE activity gels, putatively designated *E. coli* K12 peptidase E.

3.2.2.12 Growth Rate Comparisons for Aspartyl-Peptidase Carrying Strains on Different Aspartyl-Peptide Substrates

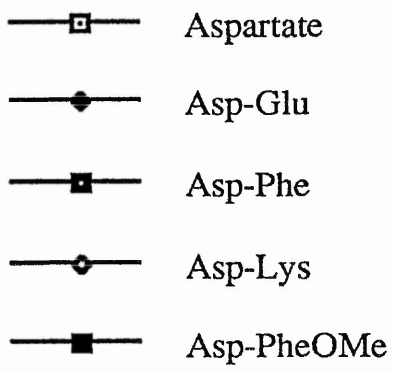
Quantitative growth tests were carried out as part of an investigation into the roles of the aspartyl peptidases in cellular metabolism, and in order to further study possible differences in substrate specificities between peptidase B and the putative *E. coli* K12 peptidase E, as not all L-α-aspartyl peptide served as substrates for the gel-based assays. Strain PN31 (*pepE*$^+$) and strain PN56 (*pepB*$^+$ *pepE*$^+$) were grown up in liquid M9 medium supplemented with different N-terminal aspartyl peptides at a final concentration of 3mM (this concentration of peptide gave

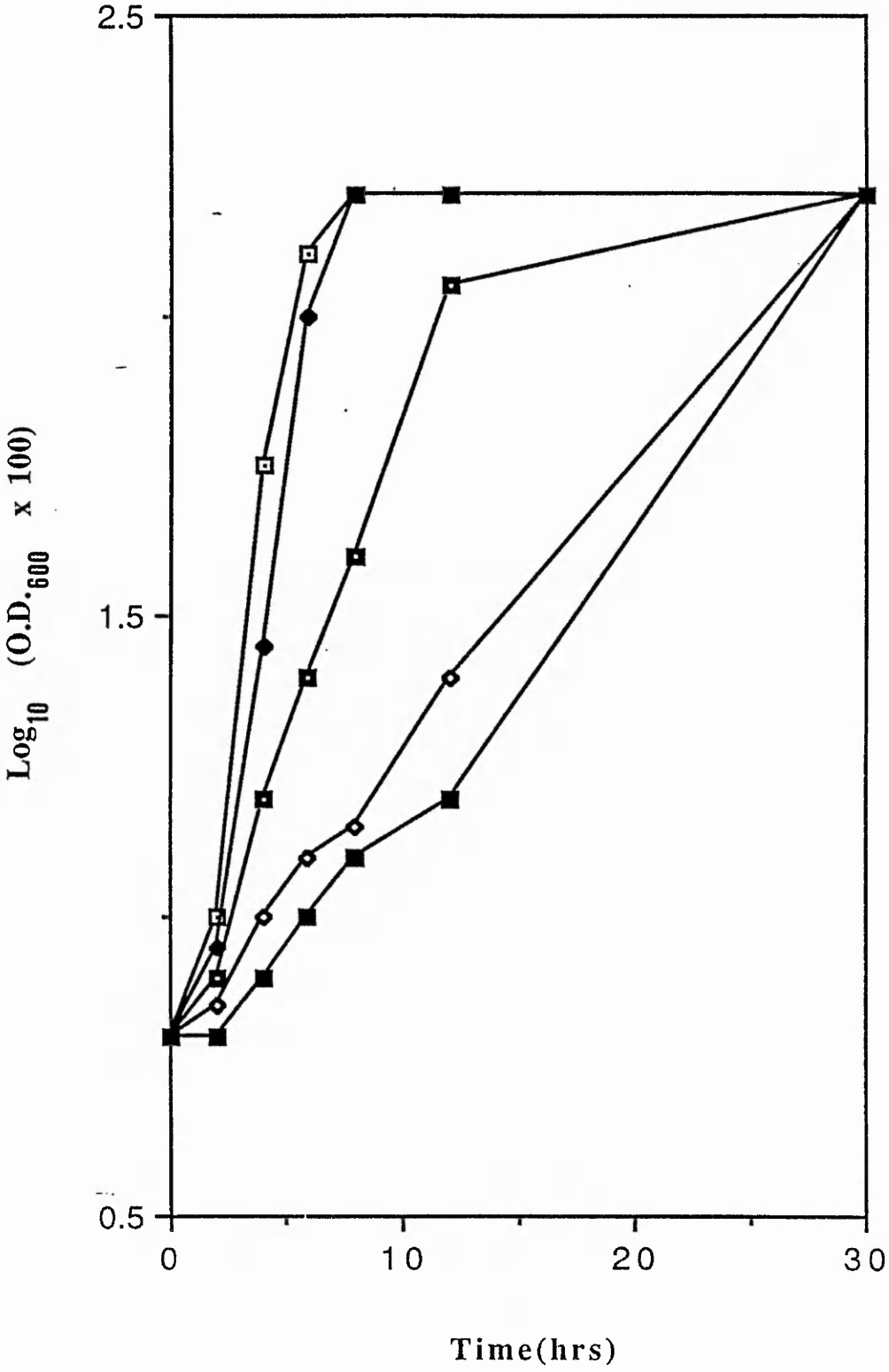
rapid growth rates facilitating data collection), providing the sole source of required aspartate. Samples of cultures were taken at time intervals and graphs plotted of optical density (O.D. 600nm) against time (Fig. 5). Graphs for strains PN31 and PN56 were virtually identical with each peptide providing no evidence for any differences in aspartyl-peptide specificity between the 2 peptidases B and E. The rate limiting step in peptide utilisation has been shown to be peptide uptake and transport in a number of bacterial systems including E. coli K12 (Payne & Bell, 1979) indicating that the growth data did not preclude differences in aspartyl-peptide specificities between the 2 peptidases. Peptidase phenotype did not however appear to have any signficant effect on growth rates in these experiments.

The variation in growth rates observed with both strains on the different peptide substrates probably reflects differences in the rates of peptide uptake in these strains rather than differences in rates of peptide hydrolysis. Both strains grew more rapidly on the amino acid aspartate than aspartyl peptides with Asp-PheOMe giving slowest growth rates and Asp-Glu giving maximal growth rates.

Growth rates of both strains increased with increasing concentrations of aspartate or aspartyl peptide and graphs showing specific growth rate versus peptide concentration

Figure 5. Growth Curves For E.coli PN31 Supplied With Different Aspartyl Peptides



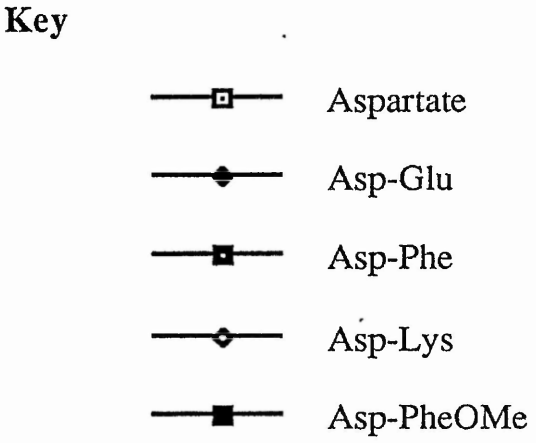

2.5
1.5
0.5
0
10
20
30
Log10 (O.D.600 x 100)
Time(hrs)

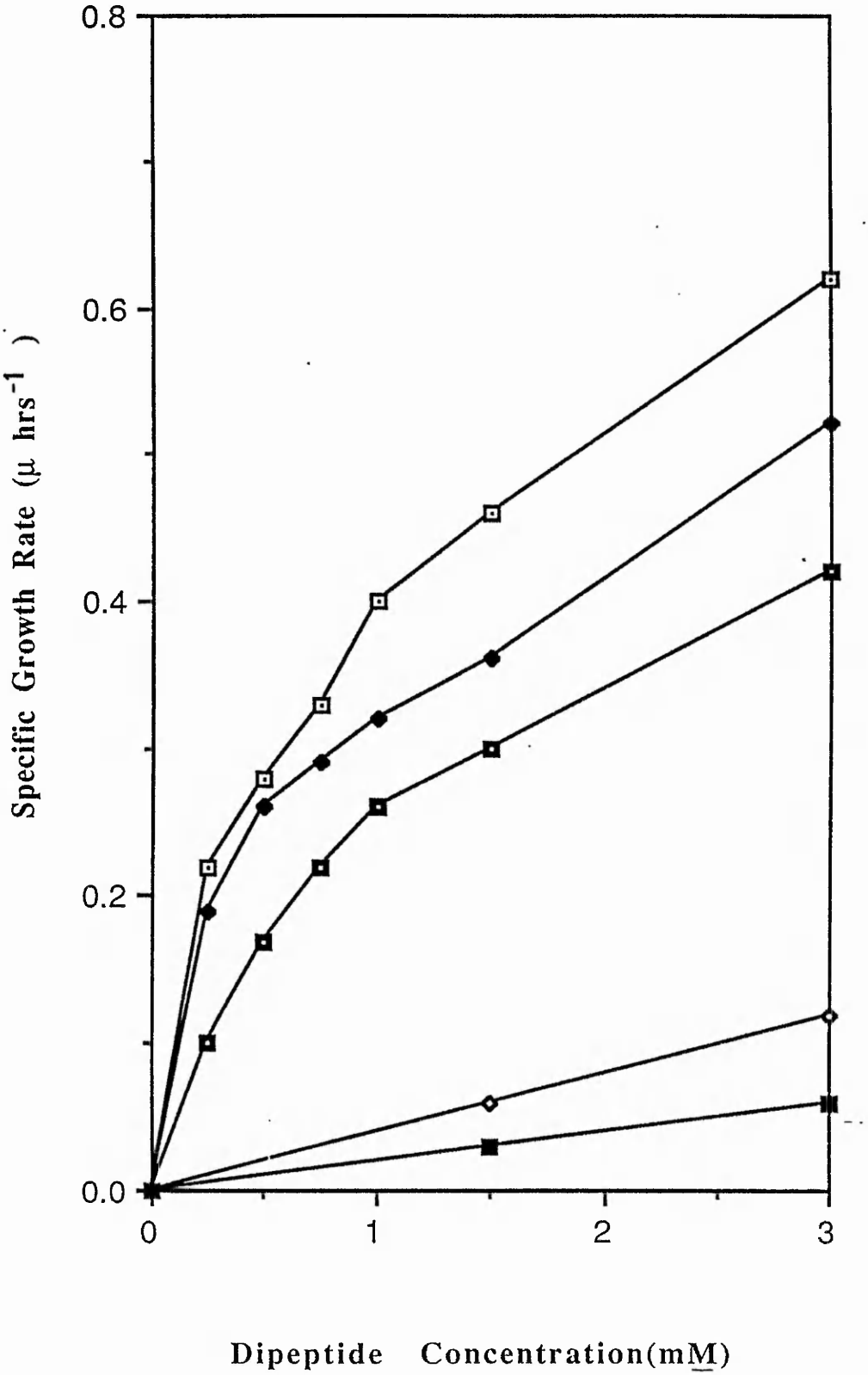
were similar for both strarins (Fig. 6). Peptidase phenotype therefore appeared to have no effect on growth rates for either strain at any tested concentration.

3.2.2.13 Effects of Metal Ions, pH and Other Factors on Growth Rates of Aspartyl Peptidase Carrying Strains

Metal ions have been shown to act as co-factors for a number of different peptidase enzymes (Yang & Somerville, 1980) and early experiments on cell extracts from the peptidase B carrying strain CM17 (section 3.2.2.2) had shown the activity of this enzyme to be stimulated by Mn^{2+} ions. Was this Mn^{2+} activation of physiological significance? Would any Mn^{2+} stimulated effect be observed on growth rates for a *pepB*$^+$ strain under appropriate conditions? The strains PN31 (*pepE*$^+$) and PN56 (*pepE*$^+$ *pepB*$^+$) were grown up in liquid M9 medium supplemented with divalent cations or the metal ion chelator EDTA at a concentration of 100 μM, and the dipeptide Asp-Phe or aspartate at concentrations of 3mM to provide a source of required aspartate. Cells were inoculated from overnight minimal medium grown cultures (M9 plus appropriate aspartate source) after washing twice with sterile 0.85% saline to remove any possibility of growth effects derived from metal ions found in the normal washing phosphate buffer. Analysis of calculated specific growth rates and doubling times showed that addition of the metal ions Co^{2+}, Mn^{2+} or Zn^{2+} had no effect on the growth rates of

Figure 6. Growth Rates Of E.coli PN31 On Different Concentrations Of Aspartyl Peptides

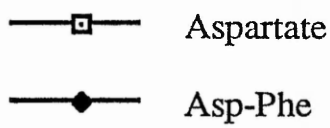


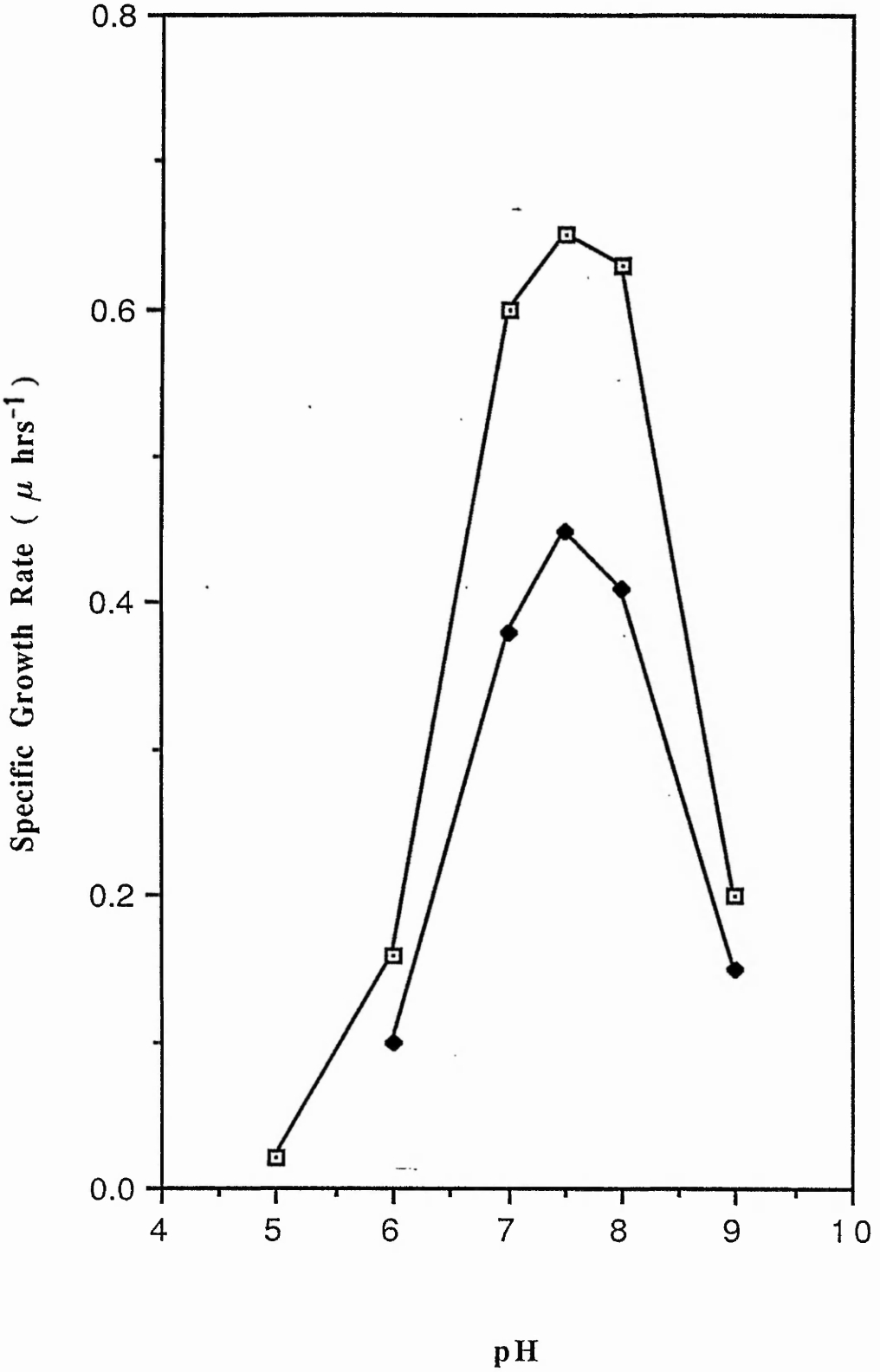

0.8
0.6
0.4
0.2
0.0
0
1
2
3
Specific Growth Rate (μ hrs-1)
Dipeptide Concentration(mM)

either aspartyl peptidase carrying strains with either aspartate or Asp-Phe as growth substrate, when comparison was made with non-metal ion supplemented media (data not shown). The presence of 100 μM EDTA in the salts medium did produce a small decrease in growth rate with both substrates, with a similar effect on both cultures. Metal ions have a variety of roles in bacterial metabolism and as there was no observed difference in metal ion or EDTA effect between the 2 strains it was not possible to propose any link between these factors and peptidase B activity in growing cells. The metal ion chelating agent EDTA could effect growth by denying cells access to a variety of metal ions required for cellular metabolism.

Identical effects were observed on both strains and with amino acid and peptide substrates when the pH of the medium was varied. For both strains and with both substrates, growth rates were optimum at an initial pH of around pH 7.5 with growth rates declining rapidly below pH 6.0 and above pH 8.0 (Fig. 7). Aspartyl-peptide hydrolysing enzymes, like other enzymes are undoubtedly effected in their activity by changing pH but specific effects could not be correlated with aspartyl peptidase activity using this data. Intracellular pH is tightly controlled and varies in *E. coli* K12 at around 0.1 pH units with each external pH change of 1.0 unit (Booth 1985).

Figure 7. Effect Of Growth Medium pH On Growth Rates Of E.coli PN31 Supplied With Different Sources Of Aspartate




0.8
0.6
0.4
0.2
0.0
4
5
6
7
8
9
10
Specific Growth Rate (μ hrs-1)
pH

Preliminary growth tests were carried out to examine regulatory mechanisms for aspartyl-peptidase biosynthesis. Cells from strains PN31 and PN56 were grown to exponential phase in M9 minimal medium containing either 3mM Asp-Phe, Asp-Lys, or Asp as sole sources of aspartate. Cells were washed in phosphate buffer and inoculated into growth flasks each containing one of the 2 aspartyl peptides. Analysis of the specific growth rates and doubling times for both strains demonstrated that the composition of the pre-inoculation medium did not effect growth rates on the 2 dipeptides, Asp-Lys and Asp-Glu nor was there any detectable lag period after inoculation from any of the different pre-inoculation media. This provides preliminary evidence that aspartyl-peptide hydrolysing enzymes may be synthesised constitutively.

3.2.2.14 Comparison of Growth Rates and Lag Times for Peptidase Carrying Strains after Transfer of Cultures from Rich to Minimal Medium

A number of studies have provided direct evidence for the role of bacterial peptidases in protein turnover (Yen et al., 1980b; Reeve et al., 1984). In order to investigate the possible role of the aspartyl peptide hydrolysing enzyme peptidase B in protein turnover, lag times and specific growth rates were determined for peptidase carrying E. coli K12 strains after transfer of exponential phase cultures from nutrient broth to aspartate supplemented minimal medium (Table 8). Strain PN31 deficient in 4 broad specificity

Table 8. Effect of Peptidase Genotypes on Lag Times and Growth Rates for *E. coli* K12 Peptidase Mutants

Strain	Genotype				Lag Time (mins)	μ^a (hrs^{-1})	g^b (hrs)
	$pepA^+$	$pepB^+$	$pepN^+$	$pepD^+$			
PN31	-	-	-	-	165	0.59	1.17
PN53	+	-	-	-	75	0.7	0.99
PN56	-	+	-	-	110	0.60	1.12
PN59	-	-	+	-	75	0.68	1.02
PN65	-	-	-	+	105	0.65	1.07

[a] μ is the specific growth rate for each strain

[b] g is the mean generation time (doubling time)

peptidases including peptidase B but carrying the E. coli K12 aspartyl peptidase E, shows the longest lag period though the specific growth rate of this strain is similar to that of the peptidase carrying strains. Strains each carrying a different broad specificity peptidase, including strain PN56 (pepB$^+$) show a marked reduction in the observed lag period when compared to strain PN31. The transfer of culture from nutrient to minimal lag leads to the synthesis of novel proteins within the cell (Yen et al., 1980b) and adaptation to new conditions. This process requires degradation of cellular proteins to component amino acids, the terminal steps of which are catalysed by peptidases. The absence of a particular peptidase may deny the cell access to amino acids required for the synthesis of new proteins or may cause accumulation of toxic peptides. The data from this experiment suggests a role for the aspartyl peptide hydrolysing peptidase B either in protein turnover or in the removal of toxic peptides.

3.2.3 Studies on Aspartyl Peptide Utilisation in Other Microorganisms

3.2.3.1 Peptidase Utilisation by Bacteria and Fungal Isolates

Qualitative growth tests were carried out to determine whether aspartame and other peptides could be used by differential bacterial and fungal strains as sources of required nutrients. All phenylalanine containing peptides

tested, Phe-Gly, Phe-Phe, Phe-Gly-Gly, Phe-Leu and the aspartyl peptides Asp-PheOMe and Asp-Phe were found to serve as sources of phenylalanine for the B. subtilis phenylalanine auxotroph strain BR-17. This strain, as well as E. coli NCTC 1896 was able to utilise Asp-PheOMe and Asp-Phe as sole sources of nitrogen in otherwise nitrogen deficient medium.

Fungal strains differed markedly in their ability to utilise different nitrogen sources, and as the extent of this growth could be assessed by observation of inoculated plates, the amount of growth was estimated for each different nitrogen source with each fungal strain (Table 9). All strains tested could grow on minimal fungal growth media containing the amino acid aspartate and phenylalanine methyl ester, an amino acid ester, as sole sources of nitrogen. Fewer strains would utilise aspartame (Asp-PheOMe) as sole source of nitrogen although fungal isolate PD0407 grew in abundance on Asp-PheOMe; far more efficiently than on other nitrogen sources. Isolates L-1, C-27 and the strain Aspergillus nidulans showed no growth on this substrate. The inability of these strains to grow on Asp-PheOMe may represent an inability to transport the peptide into the organism as has previously been reported for some peptides with N. crassa (Wolfinbarger & Mazluf, 1975). Ammonium and nitrate ions (NO_3-) were used by most organisms as sources of required nitrogen. Interestingly, certain fungal strains

Table 9. Growth of Fungi on Different Nitrogen Sources

N-Source	$(NH_4)_2SO_4$	$NaNO_3$	Asp-PheOMe	Asp+PheOMe
Fungal Strain				
Aspergillus nidulans	+/-	-	-	+++
Neurospora tetrasperma	+	++	++	++
PDO 407	++	+	++++	++
L-1	+	+	-	+
C27	++	++++	-	++++
AV03C	-	+++	+++	+++
AV0413	++	++++	+	++++

- indicates no growth. Growth scored on a + (small amount of growth) to ++++ (abundant growth) scale.

Nitrogen sources were present in media at equivalent molar concentrations.

gave different coloured growth dependent on the particular nitrogen source and the time of incubation on plates (Fig. 8). Fungal isolate AV04B produced yellow mycelium as well as excreting/secreting a yellow pigment into the medium after 2 days growth at 30°C on $NaNO_3$, but after extended incubation for 5 days, the culture became grey-green in colour, probably due to culture sporulation. This same isolate gave a grey-green culture on amino acid or Asp-PheOMe containing medium, and cream-white growth on NH_4^+ supplemented medium. Isolate AV03C produced grey-green growth on all nitrogen sources except $NH4^+$ where culture was cream-white, whereas isolate C27 gave cream-white culture on all nitrogen sources except for $NaNO_3$.

3.2.3.2 Polyacrylamide Gel Electrophoresis and Peptidase Activity Staining of Cell Extracts from Bacterial and Fungal Strains

The diversity of organisms in which aspartyl-peptidases might be found was investigated by analysis of aspartyl-peptide hydrolysing PAGE activity profiles from bacterial and fungal cell extracts. All bacterial cell extracts tested showed bands of aspartyl-peptide hydrolysing activity on polyacrylamide gels (Fig. 9), with both Asp-PheOMe and Asp-Leu as substrates, and each Asp-PheOMe hydrolysing activity observed had a band of corresponding mobility with specificity towards Asp-Leu. The observations of multiple activities towards aspartyl-peptides was the

Figure 8. Growth of fungal strains AVO 4B on Agar Plates containing different nitrogen sources. Top, $NaNO_3$; bottom left, amino acids; bottom right NH_4^+

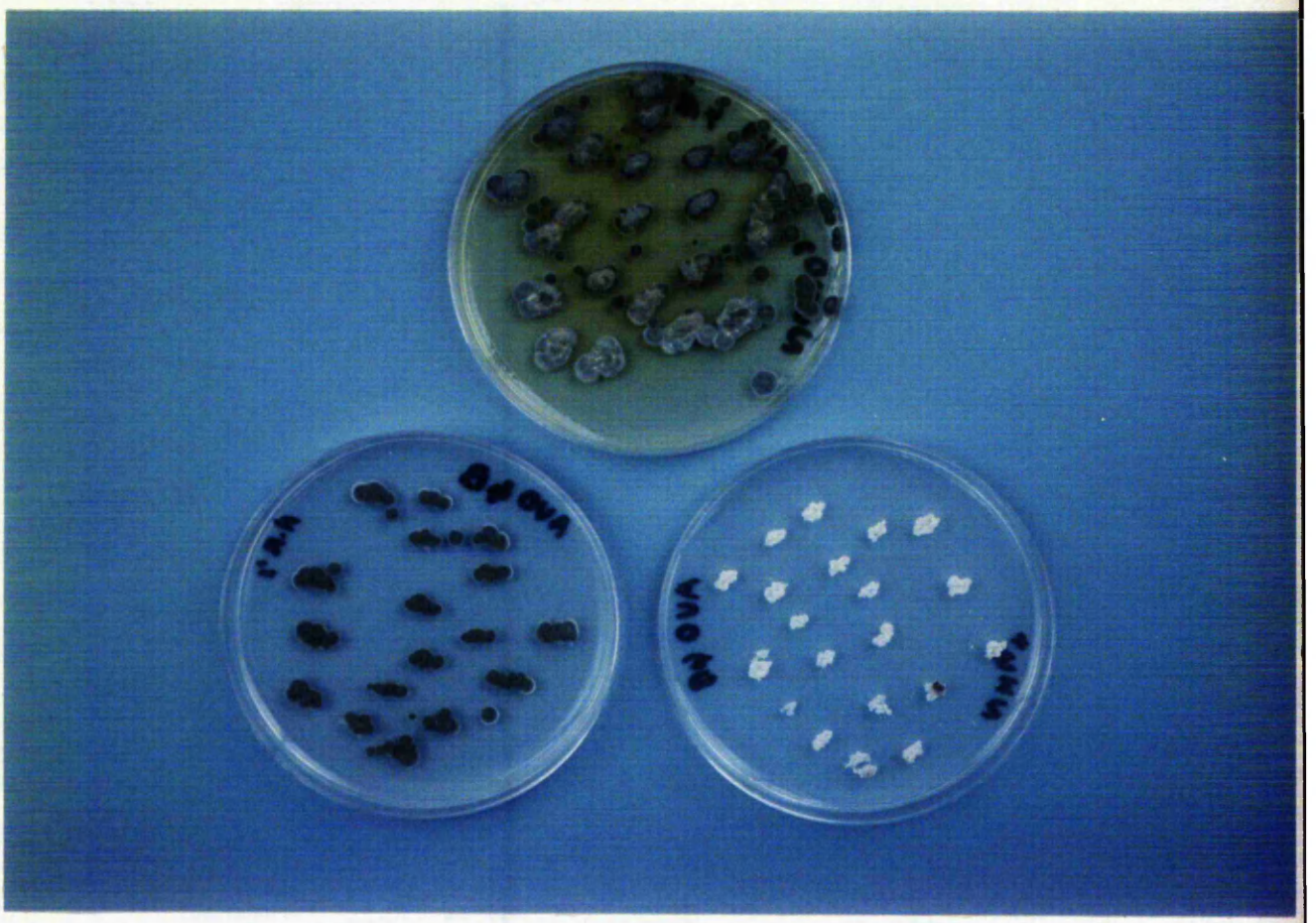
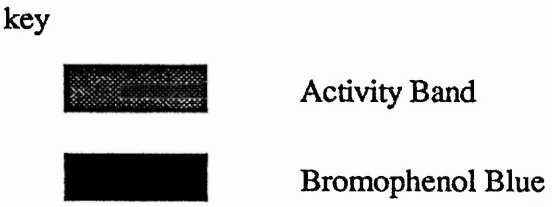
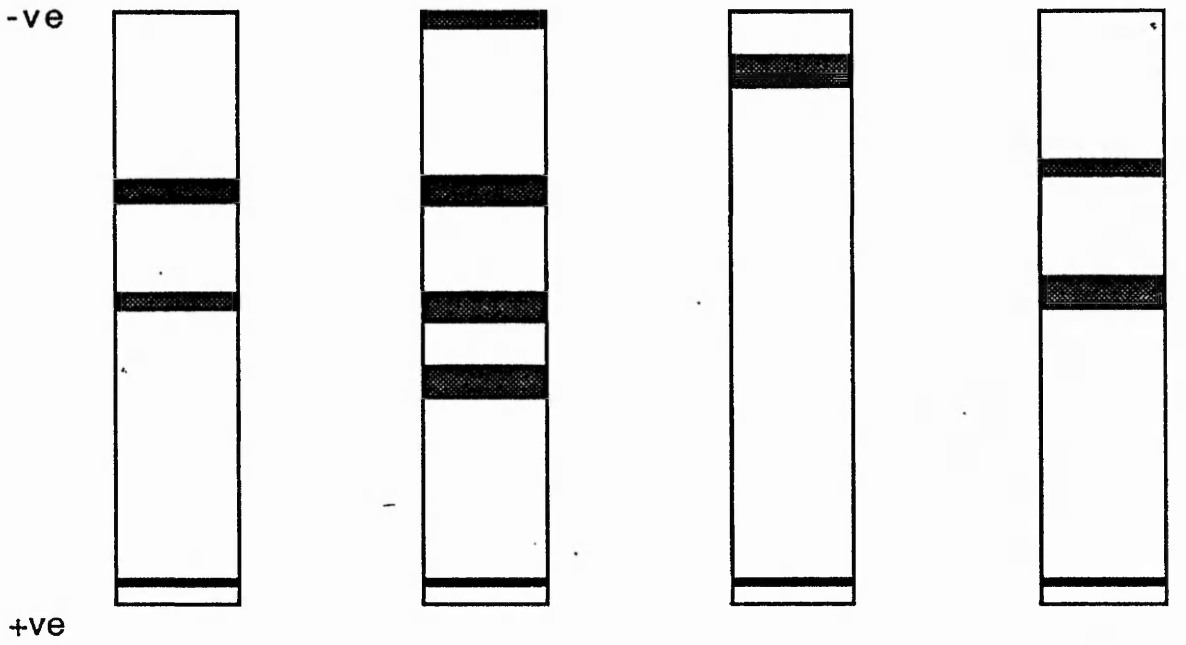


Figure 9. Aspartame / Asp-Leu Hydrolysing Activity Profiles Of Bacterial Strains

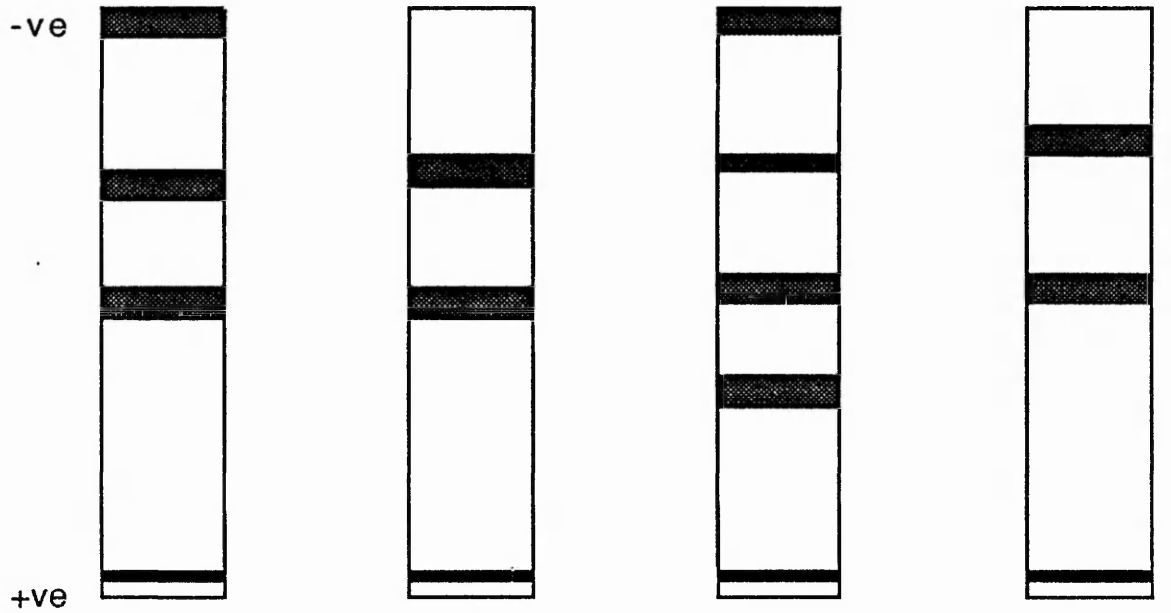


Activity Band Mobilities

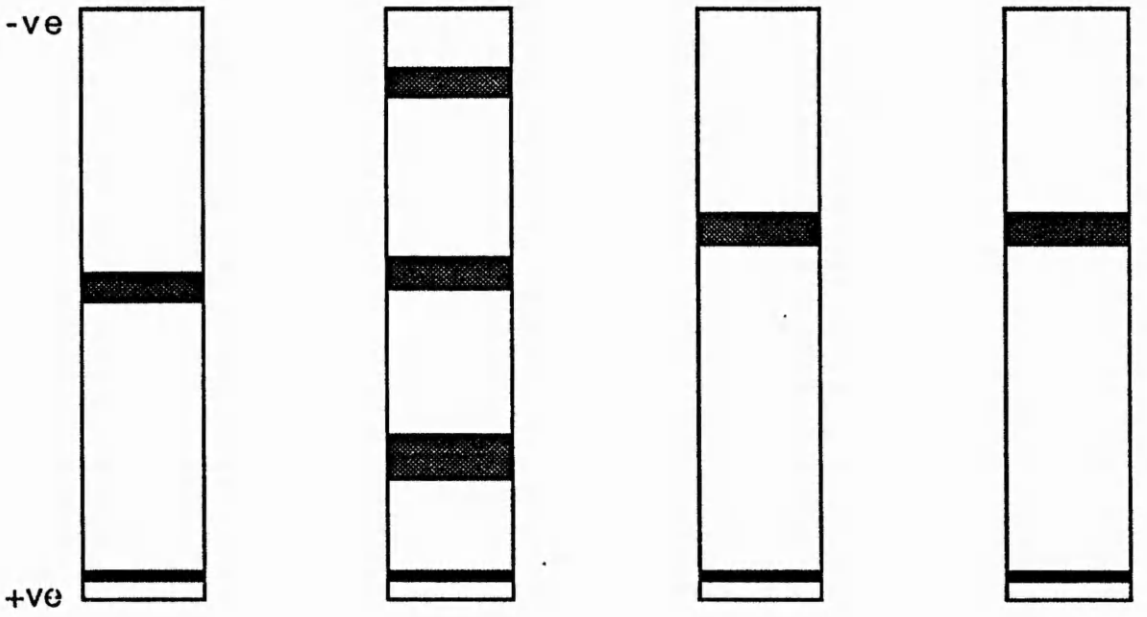
C.freundii	*0.32,0.51*
E.coli K10	*0.02,0.32,0.53,0.66*
B.subtilis BR-17	*0.1*
P.fluorescens	*0.28*
P.vulgaris	*0.02,0.3,0.52*
B.sphaericus	*0.29, 0.51*
A.calcoaceticus	*0.02,0.26,0.49,0.68*
F.denitrificans	*0.22,0.48*
Rhizobium	*0.49*
E.coli B	*0.13,0.47*
M.luteus	*0.39*
M.roseus	*0.39*
A.faecalis	*0.27,0.51,0.72*
E.coli NCTC1896	*0.29,0.48*
C.fascians	*0.26,0.57,0.68*



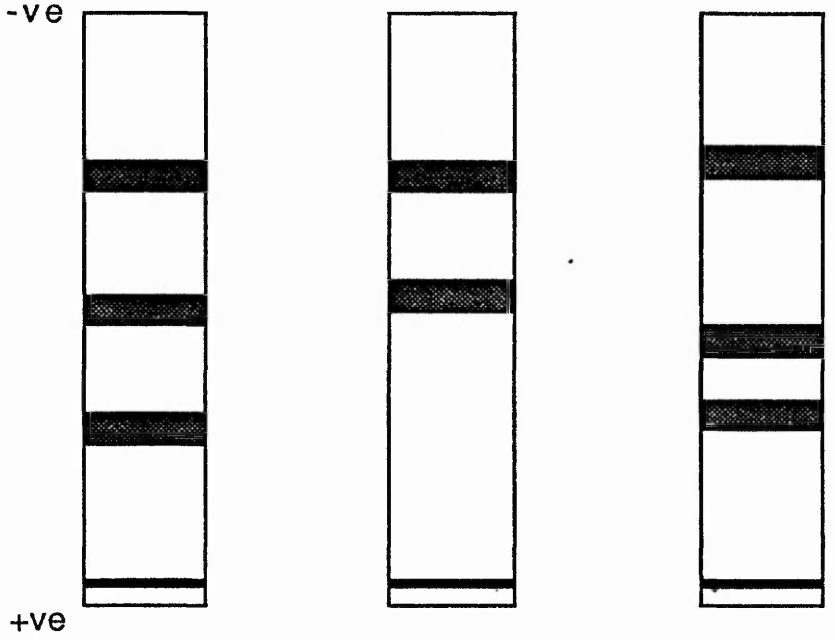
Corynebacterium freundii | *Escherichia coli K10* | *Bacillus subtilis BR-17* | *Pseudomonas fluorescens*



Pseudomonas vulgaris | *Bacillus sphaericus* | *Acinetobacter calcoaceticus* | *Flavobacterium denitrificans*



Rhizobium | *Escherichia coli B* | *Micrococcus Luteus* | *Micrococcus Roseus*



Alicaligenes faecalis | *Escherichia coli NCTC 1896* | *Corynebacterium fascians*

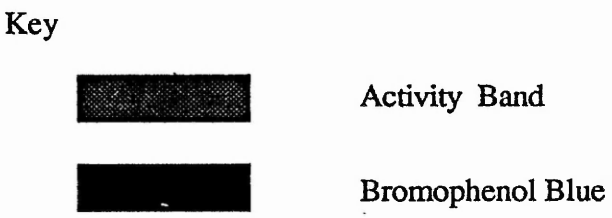
rule rather than the exception. The bacterial strains E. coli K10 and Acinetobacter calcoaceticus var anitratus both showed 4 bands of aspartyl-peptide hydrolysing activity in these assays. Mobilities of the aspartyl peptide hydrolysing enzymes differed substantially indicating differences in molecular weight, conformation, or charge.

Intracellular Asp-PheOMe/Asp-Leu degrading activities were found in all fungal cell-free extracts although as in bacterial systems the number of aspartyl peptide hydrolysing enzymes and the mobilities of these enzymes differed markedly between the different strains (Fig. 10). Strains which had been unable to utilise Asp-PheOMe as a source of nitrogen still possessed Asp-PheOMe hydrolysing intracellular activities and this evidence suggested that these peptidases were more likely to have a role in the degradation of intracellular peptides either generated by protein turnover or some other cellular process rather than in the utilisation of exogenously supplied peptides.

3.2.3.3 Polyacrylamide Gel Electrophoresis and Peptidase Activity Staining for Extracellular Aspartyl-Peptidase Activities in Bacterial and Fungal Strains

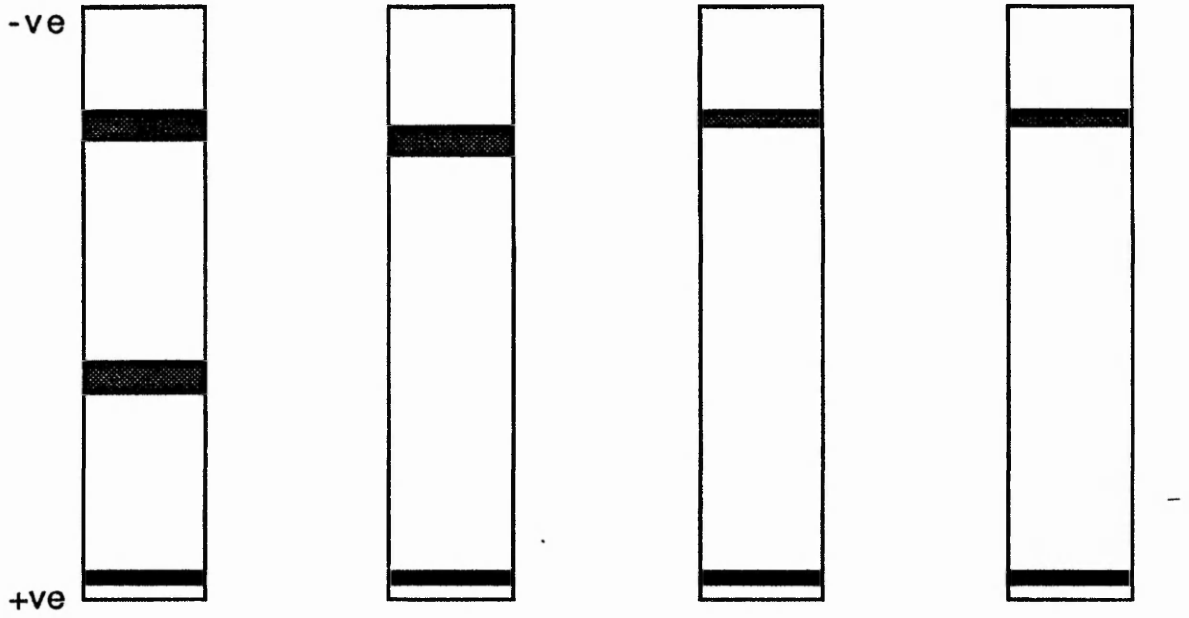
Concentrated preparations of growth media from 200 ml cultures of B. subtilis BR-17, on gel assay analysis showed evidence of single extracellular peptidase activity towards Asp-PheOMe mobility 0.04. Isolates from the fungal strains PD0407 and AV03C possessed a detectable, extracellular

Figure 10. Aspartame / Asp-Leu Hydrolysing Activity Profiles Of Fungal Strains

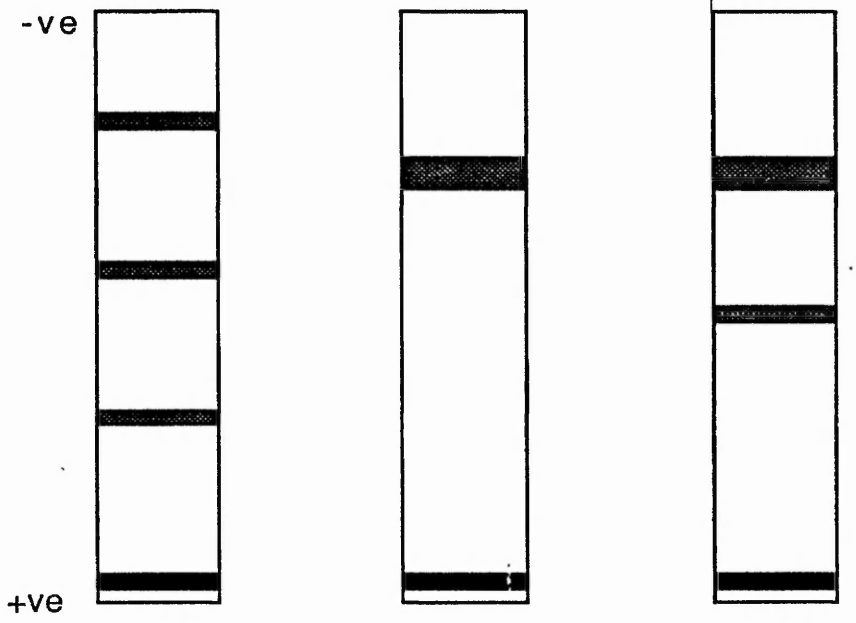


Activity Band Mobilities

A.nidulans	*0.20,0.62*
N.tetrasperma	*0.23*
PDO407	*0.19*
C27	*0.19*
L-1	*0.19,0.44,0.70*
AVO3C	*0.27*
AVO413	*0.27,0.52*



Aspergillus nidulans | *Neurospora tetrasperma* | *PDO 407* | *C27*



L - 1 | *AVO3C* | *AVO413*

Asp-PheOMe/Asp-Leu hydrolysing activity mobility 0.2 on gel assays.

3.2.3.4 Polyacrylamide Gel Electrophoresis and Activity Staining for Aspartyl-β-napthylamide Hydrolysing Activities in Bacterial Strains

In preparation for experiments aimed at purifying bacterial aspartyl-peptide hydrolysing enzymes, crude extracts from a number of different bacterial strains were quantitatively assayed for aspartyl-β-napthylamide hydrolysing activity. Activity towards this chromogenic substrate is easily assayable in biochemical assays and a peptidase capable of hydrolysing this substrate would therefore be more suitable for purification and analysis. The only bacterial strain found to possess aspartyl-β-napthylamide hydrolysing activity in sonicated crude extracts was A. calcoaceticus var anitratus. This activity had a mobility on PAGE gels of 0.02 and was coincident with a band of Asp-Leu/Asp-PheOMe hydrolysing activity suggesting that the same enzyme was responsible for the hydrolysis of each of these substrates. No aspartyl-β-napthylamide hydrolysing activity was observed either on gels or on quantitative assay in crude cell extracts derived from Pseudomonas vulgaris, E. coli CM17, Pseudomonas fluorescens, Bacillus subtilis, Bacillus sphaericus, Corynebacterium freundii, Flavobacterium denitrificans, Escherichia coli K10, and a rhizobium.

3.3 Discussion

The results obtained in this chapter demonstrate that the ability to hydrolyse L-α-aspartyl peptides using peptidase enzymes, and the ability to utilise these substrates as nutrients, are commonly found in both bacteria and fungi. All microorganisms tested possessed aspartyl-peptide hydrolysing activities and all of the bacterial strains and some fungal strains could grow on aspartyl-peptides as sources of nitrogen or required amino acids. The presence of multiple peptidases capable of hydrolysing aspartyl peptides was observed in gel assays on crude cell extracts from most organisms studied, providing a common feature for most aspartyl-peptide degrading systems. *E. coli* K10 and the Gram-negative chemoheterotroph *A. calcoaceticus var anitratus* appeared to have at least 4 aspartyl-peptide hydrolysing activities.

Along with the phenomenon of peptidase multiplicity, there appears, from analysis of the aspartyl-peptidase mobilities on polyacrylamide gels, to be substantial variation in size, shape or charge of the different aspartyl peptidases, both between different genera and species and within a single microorganism. Different strains of *E. coli* K12 show different aspartyl-peptidase profiles though all strains have an activity mobility 0.5. These observations could suggest a lack of evolutionary conservation of aspartyl peptidase enzymes and enzyme systems, as well as suggesting potentially diverse evolutionary origins for the individual peptidases. The presence of multiple peptidases

with overlapping specificities would provide a genetic background against which peptidase mutations causing alterations in specificity could occur without seriously affecting cell viability.

Extracellular aspartyl-peptide hydrolysing activities were detected in 2 fungal isolates, PD0407 and AV03C, originally isolated as strains possessing high extracellular esterase activity. In previous studies carried out on Neurospora crassa, extracellular peptidase activity was detected in the growth medium towards a limited range of peptides (Wolfinbarger & Mazluf, 1974) but no aspartyl peptide was used as a substrate in these experiments. This extracellular peptidase activity could only be detected after concentration of the growth medium and prolonged incubation with peptide substrates. Extracellular aspartyl-peptidase activities were detected readily in the experiments reported in this chapter; this difference in the 2 studies may be ascribed to the use of different peptidase assay systems, the rich peptone and corn steep liquor containing rich medium used here for the growth of fungal strains which might stimulate production of extracellular peptidases, or the pre-selection in this work for fungal strains producing high extracellular esterase activity which could be directly or indirectly linked to higher levels of extracellular peptidase activity.

Two aspartyl peptide hydrolysing activities were detected in E. coli K12. Data from a previous study suggested that each of the 4 broad specificity peptidases A, B, D and N could hydrolyse the dipeptide Asp-Leu, and it was reported that a E. coli K12 strain lacking these enzymes was unable to utilise Asp-Leu as a source of required leucine (Miller & Schwartz, 1978). The results reported here directly contradict these findings. The E. coli K12 strain CM89 lacking peptidases N, A, B, D was found to utilise Asp-Leu as sole source of required leucine. Studies using gel based assays showed that only the broad specificity peptidase B and a previously unreported activity (designated E. coli K12 peptidase E due to similarities in substrate specificity with the peptidase E reported in S. typhimurium) could hydrolyse Asp-Leu. These 2 Asp-Leu hydrolysing activities would also hydrolyse Asp-PheOMe. Peptidases A, B, D and N showed no activity on gel assays towards aspartyl peptide substrates. However transductant strains carrying the pepQ allele gained the ability to utilise Asp-Pro as a source of aspartate whereas pepQ deficient strains could not, suggesting that peptidase Q can degrade Asp-Pro. The S. typhimurium peptidase E was reported to hydrolyse the Asp-Pro dipeptide (Carter & Miller, 1984), however the E. coli K12 pepE$^+$ strain PN31 was unable to utilise Asp-Pro as a source of nutritional requirements indicating that the E. coli K12 peptidase E cannot hydrolyse Asp-Pro.

A number of different strategies were used in efforts to isolate aspartyl-peptidase deficient strains but none met with

success. Concentration of cell extracts prior to gel assays, and the use of different incubation conditions for these assays did not however reveal any further peptidase activities which would account for difficulties in mutant isolation. Such additional aspartyl-peptide hydrolysing activities have been identified in S. typhimurium LT2 (Carter & Miller, 1984) and the evidence from gel analysis and microassays suggests that such additional peptidase activities may be present in E. coli K12 but are inactivated or denatured during the production of dialysed, sonicated crude cell extracts. Alternatively, if as has been proposed, aspartyl peptides are highly toxic to aspartyl-peptidase deficient strains (Carter & Miller, 1984), such deficient mutant cells might not be viable and hence would prove impossible to isolate. Furthermore it is also possible that these additional activities are associated with cell membranes removed by centrifugation at 38,000g during cell extract manufacture.

The Co^{2+} stimulated K12 activity designated aminopeptidase L, with specificity for Leu-Gly and Leu-Gly-Gly and reported to have mobility 0.28 on 7.5% polyacrylamide gels (Miller & Schwartz, 1978) was not observed in this work after gel staining of crude extract from E. coli CM17 and CM89. This activity band was reported as variably present in this earlier study although the reasons for this variability were not discussed. The absence of this activity band in the gels reported in this chapter may relate to reduced sensitivity in the disc gel system used in this

work. Alternatively different growth media and conditions used for generation of cell cultures may have affected the peptidase profiles of these strains. A similarity in mobility with the peptidase B activity band mobility 0.3 could possibly have obscured the 0.28 band, although strain CM89, deficient in peptidase B produced no activity bands with this mobility. The gel based activity assay appears to be unable to detect all peptidase activities, in the case of the work reported here, possibly certain aspartyl-peptidase activities, but also peptidase M (Miller et al., 1987), and is variable in its detection of the designated aminopeptidase L.

Quantitative growth tests on E. coli K12 peptidase deficient strains revealed that there were no significant differences in growth rates between strains producing the aspartyl-peptide hydrolysing enzyme peptidase B and those deficient in this peptidase when these strains were grown on aspartyl peptides as sources of aspartate. Growth rates were significantly different dependent on the concentration and C-terminal residue of aspartyl-peptide growth substrates but these effects were identical between the different strains. Preliminary growth test data suggested that aspartyl-peptidase activities might be synthesised constitutively. The peptidase B enzyme appeared to have some role in the process of adaptation and protein turnover accompanying the switch of E. coli K12 cultures from rich to minimal medium.

The only bacterial strain found to contain an aspartyl-β-napthylamide hydrolysing activity in crude cell extracts, was Acinetobacter calcoaceticus var anitratus. This non-fastidious microorganism can be found naturally in soil, water and sewage (Bergey, 1984) and can use a wide range of carbon and nitrogen sources. It was decided to study further the ANA hydrolysing enzyme as it appeared to be able to hydrolyse aspartyl peptides as well as the easily assayable ANA. The K12 aspartyl-peptidases B and E were also selected for further studies which are described in the following chapter.

CHAPTER 4 THE PURIFICATION AND CHARACTERISATION OF ASPARTYL PEPTIDE HYDROLYSING ENZYMES

4.1 Introduction

Following the initial identification of a number of aspartyl-peptide hydrolysing enzymes it was decided to study the properties of some of these peptidases. Experiments were carried out to investigate the factors affecting aspartyl-peptidase activity, the substrate specificities of these enzymes, their physical properties and other characteristics. Such experiments would help in elucidating the mechanisms of catalysis employed by these enzymes and possibly provide information on the role of these enzymes in the microbial cell.

The 2 *E. coli* K12 aspartyl-peptide hydrolysing enzymes identified in the previous chapter, peptidase B and the designated peptidase E were chosen for study, as well as the aspartyl-β-napthylamide (ANA) hydrolysing activity, identified in the bacterium *A. calcoaceticus* using gel-based assays. As *E. coli* K12 and its peptidases are comparatively well characterised and well studied, simple methods were available for the separation and analysis of its aspartyl-peptidases; the previous studies carried out on other *E. coli* K12 peptidases would provide a basis for comparative analysis of the *E. coli* K12 aspartyl-peptidase. In addition, analysis of the *E. coli* K12 peptidases B and E would allow comparison of aspartyl-peptidases within the same organism. The *A. calcoaceticus* ANA hydrolysing enzyme was chosen for study mainly as this activity was amenable to

simple quantitative assay due to the chromophoric nature of ANA degradation products. Study of this enzyme would enable comparison of peptidases from different bacteria.

Purification of the 3 aspartyl-peptidases under investigation was essential in order to allow separation of these enzymes from possible contaminating peptidases, and in the case of the E. coli K12 peptidases to separate peptidases B and E themselves. The E. coli K12 strain PN31 and PN56 were used for the purification of the K12 peptidases. Strain PN56 carries both aspartyl-peptide hydrolysing enzymes but no other broad specificity peptidases whereas strain PN31 carries only E. coli K12 peptidase E.

The procedure adopted for purification of all 3 peptidases involved 3 steps and was based on that used for the purification of the S. typhimurium peptidase E (Carter & Miller, 1984). After large scale growth of bacterial cultures in shake flasks, crude extracts were generated using a French Pressure Cell. These extracts were subject to an initial purification using ammonium sulphate fractionnation, a method which selectively precipitates proteins on the basis of their molecular charge. Activity containing fractions from this procedure were subject to ion-exchange column chromatography using the anionic exchange resin DE52 providing further purification of

peptidases on the basis of molecular charge. Molecular mass dependent purification was then carried out by gel filtration chromatography on a Sephadex G200 column. This method was also used for estimation of peptidase molecular mass. Pooled activity containing fractions obtained after these 3 stages of purification were used in biochemical assays. The molecular mass values for E. coli K12 peptidase holoenzymes were confirmed by analysis of mobilities on different concentration polyacrylamide gels (Hedrik & Smith, 1968).

Fractions containing E. coli K12 peptidase B or peptidase E activity were initially identified after each purification step using gel based assays or a qualitive peptidase microassay (Carter & Miller, 1984) with the peptidase B substrate Leu-Gly and the aspartyl-peptide Asp-Leu as assay substrates. Peptidase B and peptidase E activities were quantified by measuring the release of leucine from the dipeptides Leu-Gly and Asp-Leu respectively after samples of activity containing fraction were added to assay mixtures under standard conditions. Leucine was determined fluorimetrically after derivatisation of product mixtures with O-phthalaldehyde and separation of derivatised products using High Pressure Liquid Chromatography (Griffin et al., 1982). The activity of the ANA hydrolysing peptidase containing fractions were determined after each purification step by following the release of napthylamine

from ANA as shown by an increase in the optical density of reaction mixtures at 340 nm (Lee et al., 1971).

Quantitative assays were carried out using partially purified peptidases from ammonium sulphate fractionnation, ion-exchange chromatography and gel filtration as these fractions were devoid of detectable contaminating peptidase activities and contained sufficient activity to enable a large number of assays to be carried out. Aspartyl tripeptides and D-aspartyl peptides were not tested as assay substrates due to their expense and unavailability. Each peptidase was characterised with respect to stability under varying conditions, inhibitor effects, optimal pH, metal ion effects and substrate specificities using both qualitative and quantitative assays. The acquisition of this data enabled comparisons of the different peptidases.

4.2 Results

4.2.1 Separation of O-pthalaldehyde derivatised amino acids and peptides by reversed-phase HPLC

Product mixtures generated by aspartyl-peptidase catalysed hydrolysis of peptide substrates were derivatised with O-pthalaldehyde. These derivatised mixtures were separated by HPLC and fluorescence of separated components determined. Elution times were determined for amino acid and peptide derivatives using control samples of each moiety loaded onto the HPLC column (Table 10). Precise times were

Table 10. Elution times of OPA-derivatised amino acids and peptides separated by reversed-phase HPLC

Amino acid/Peptide	Elution time (mins)
Aspartate	3.16
Glutamate	3.42
Glycine	11.25
Taurine	13.94
Alanine	14.69
Tyrosine	14.85
Valine	18.92
Phenylalanine	19.19
Leucine	19.52
Lysine	19.84
PheOMe	——— [a]
Asp-Glu	3.28
Asp-Gly	4.60
Asp-Leu	10.81
Asp-Ala	11.8
Asp-Val	11.91
Asp-Phe	12.02
Asp-Lys	12.80
Asp-Tyr	12.81
Asp-PheOMe	19.75
ANA	——— [a]
Leu-Gly	19.8

[a] No elution peaks were identified representing ANA or PheOMe.

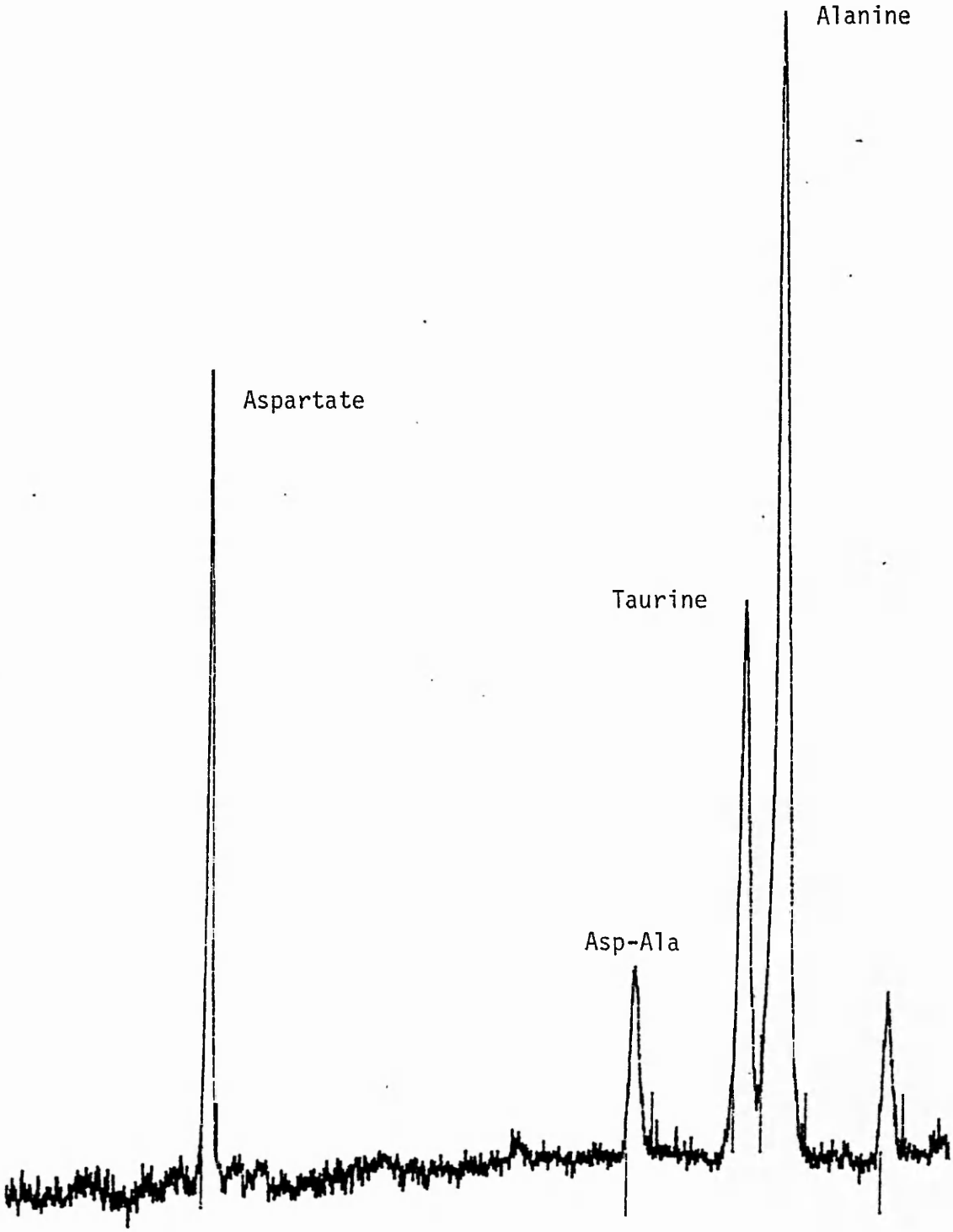
determined as averages of elution times from a number of different separations. Almost all amino acid and peptide derivatives were amenable to separation and analysis by this method enabling accurate quantitative assays to be carried out, although CBZ-Asp could not be derivatised with O-pthalaldehyde due to the absence of a free N-terminal group and no fluorescent peak could be identified representing ANA or PheOMe. Peaks representing unidentified contaminant species were often observed and in some cases double peaks were observed where a single amino acid or peptide derivative species was expected. The accompanying figure (Fig. 11) shows the separation of a product mixture derived from E. coli K12 peptidase E catalysed hydrolysis of Asp-Ala.

4.2.2 Purification of Aspartyl-Peptide Hydrolysing Enzymes

4.2.2.1 Preparation of Cell Extracts

10 dm^3 cultures of E. coli K12 strain PN31 (carrying peptidase E), PN56 (carrying both peptidases B and E), and A. calcoaceticus were grown to early stationary phase in rich nutrient broth medium in shake flasks. Cells were harvested by centrifugation, washed and resuspended in 0.05M Tris-HCl pH 7.5 prechilled at 4°C. All subsequent operations during purification were performed at 4°C in order to maintain enzyme activity, and pH 7.5 buffer was used to ensure that peptidase proteins had a substantial negative charge and could be well separated by ion-exchange chromatography. The cells in each suspension were broken in

Figure 11. Separation using reversed phase HPLC of OPA-Derivatised product mixture from E. coli K12 Peptidase E catalysed hydrolysis of Asp-Ala



the French Pressure Cell and after dialysis overnight against 0.05M Tris-HCl pH 7.5 to remove contaminating amino acids and peptides, PN31 and PN56 cell extracts were assayed qualitatively using the peptidase activity stain on non-denaturing gels to confirm the presence of peptidase B and peptidase E activities. Their presence having been confirmed, PN31 and PN56 extracts were assayed quantitatively for enzyme activities using the assay substrates Leu-Gly and Asp-Leu, as well as for protein using the Lowry assay. Peptidase E (Asp-Leu hydrolysing) activity in Strain PN31 was determined at 13 nanomoles leucine released min^{-1} mg $protein^{-1}$ (0.013 units mg $protein^{-1}$). Peptidase B (Leu-Gly hydrolysing) activity in strain PN56 was determined at 11 nanomoles leucine released min^{-1} mg $protein^{-1}$ (0.011 units mg $protein^{-1}$). The specific activity of the A. calcoaceticus ANA hydrolysing peptidase crude cell extract was assayed and determined at 27 nmoles napthylamine released min^{-1} (0.027 units mg $protein^{-1}$).

4.2.2.2 Ammonium Sulphate Fractionnation of Cell Free Extracts

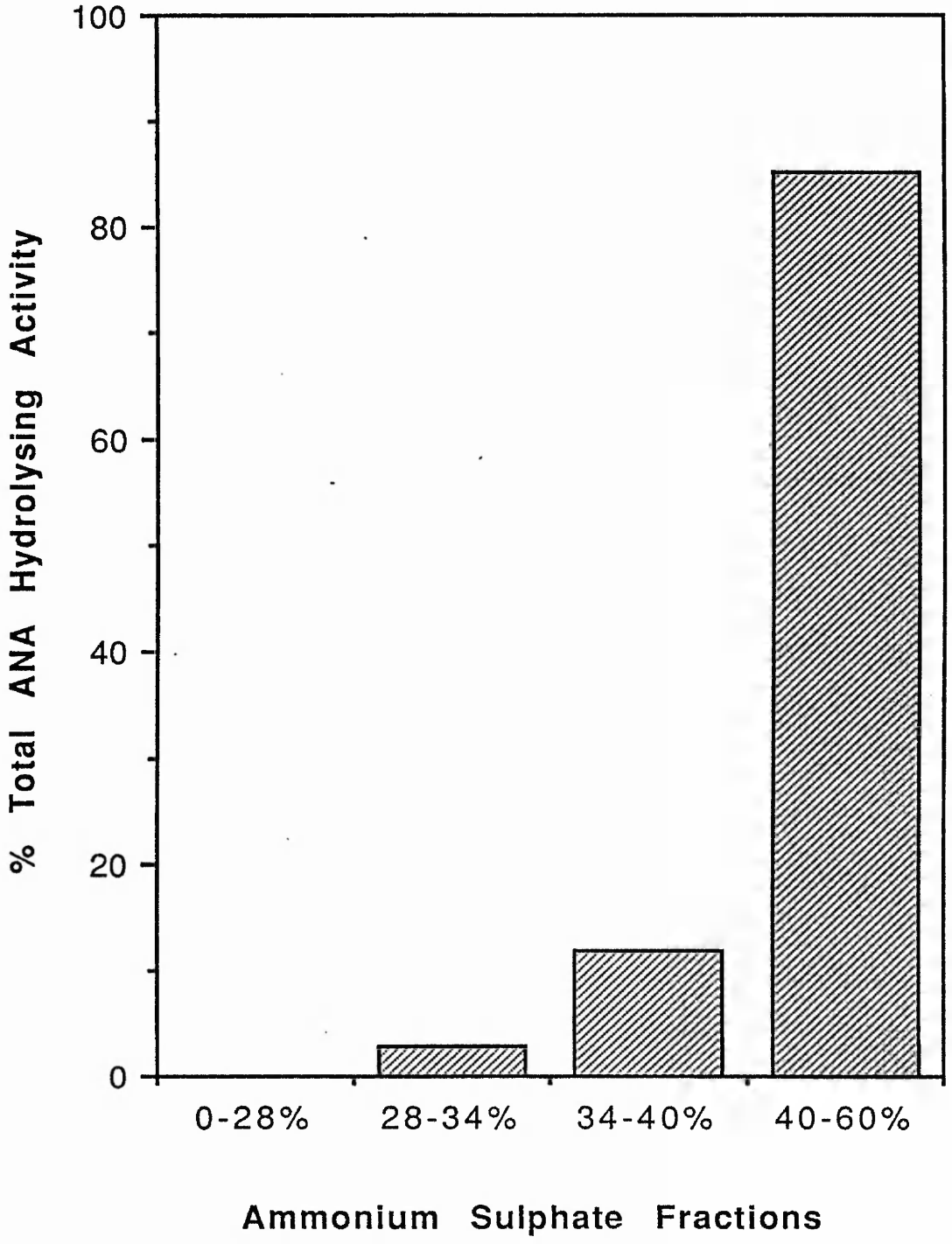
Preliminary experiments were conducted to determine the range of ammonium sulphate concentrations over which peptidase activities precipitated out. Ammonium sulphate cuts were carried out on samples of cell-free extracts in the ranges 0-28%, 28-34%, 34-40% and 40-60%. Following dialysis against 0.05M Tris-HCl pH 7.5 to remove

contaminating ammonium sulphate, fractions were assayed for protein and peptidase activities. Ammonium sulphate fractions obtained from the E. coli PN56 cell-free extract were separated on non-denaturing polyacrylamide gels and stained for activity towards the dipeptides Asp-Leu and Leu-Gly. Ammonium sulphate fractions from the A. calcoaceticus fractions were assayed for hydrolysis of ANA.

Peptidase activity bands representing peptidases B and E were only observed on PAGE gels on which samples of the 40-60% PN56 ammonium sulphate fraction had been loaded. Similarly 85% of recovered ANA hydrolysing activity was observed in the 40-60% ammonium sulphate fraction with substantially lower levels in other fractions (Fig. 12). Approximately 35% of total cell protein from all ammonium sulphate fractions was found in the 40-60% ammonium sulphate fraction of strain PN56 whereas 39% of A. calcoaceticus ammonium sulphate fraction precipitated protein was found in the equivalent 40-60% fraction.

Only 75-80% of the protein present in the total crude extracts was recovered in all ammonium sulphate fractions for both E. coli and A. calcoaceticus. In the case of A. calcoaceticus the total recovery of ANA hydrolysing activity was also around 75% of that present in the total crude cell extract suggesting that activity and protein were

Figure 12. Distribution Of A.calcoaceticus ANA Hydrolysing Activity After Ammonium Sulphate Fractionnation



probably lost due to adhesion to centrifuge tubes and other vessels rather than enzyme denaturation during precipitation. The total recoveries of PN56 peptidase activities were not determined in these preliminary experiments.

Using the results from the preliminary studies as a basis for large scale enzyme purification, 50 mls of crude cell-free extracts from each organism were subjected to a 40-60% ammonium sulphate cut. Precipitated protein was resuspended in 10 mls of 0.05M Tris-HCl pH 7.5 and dialysed overnight at 4°C against the same buffer. Fractions were assayed for peptidase activity and protein concentration. Specific activities were determined at 16 nmoles leucine released min^{-1} mg $protein^{-1}$ (0.016 units mg $protein^{-1}$) and 17 nmoles leucine released min^{-1} mg $protein^{-1}$ (0.017 units mg protein) for PN56 peptidases B activity and PN31 peptidase E activity respectively indicating purification factors of 1.5 and 1.3 for these enzymes. The specific activity for the ANA hydrolysing enzyme in the A. calcoaceticus fraction was determined at 50 nmoles napthylamine released min^{-1} mg $protein^{-1}$ (0.05 units mg $protein^{-1}$), indicating a purification factor of 1.9 for the 40-60% ammonium sulphate fractionnation of this enzyme.

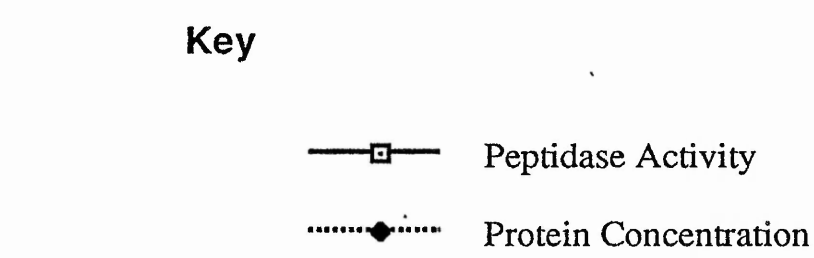
4.2.2.3 Ion-Exchange Chromatography

A suitable volume of dialysate (5mls) from ammonium sulphate

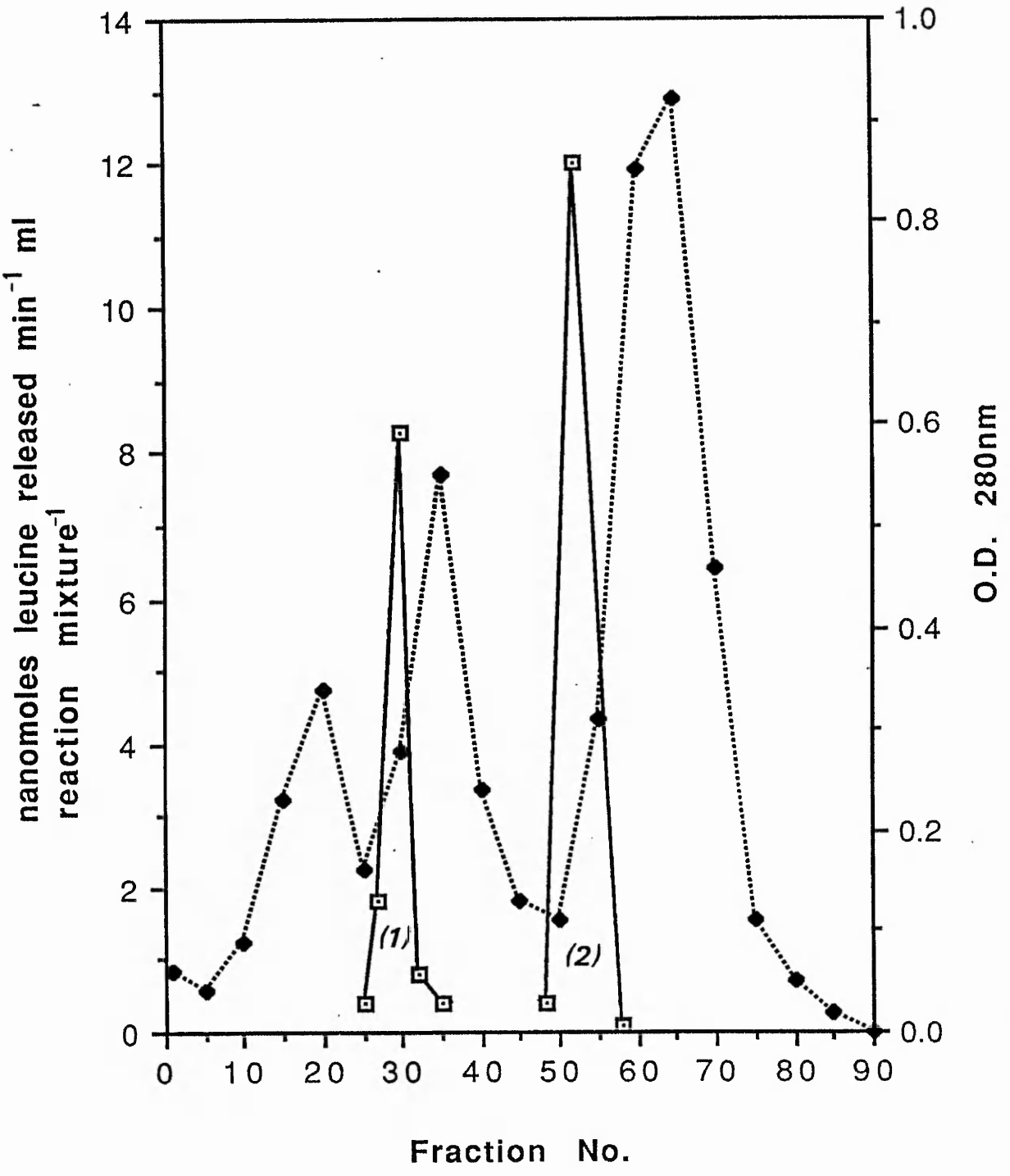
fractionnation was applied to a column of the anionic DEAE-cellulose, DE-52, which had been pre-equilibrated with 0.05M Tris-HCl pH 7.5. One column volume of the latter buffer was allowed to pass through the column before the linear ionic strength gradient of 0-0.5M NaCl, created using 0.05M Tris-HCl pH 7.5 and 0.5M NaCl, was connected and the collection of fractions commenced.

6 ml fractions collected from the PN31 and PN56 dialysed 40-60% ammonium sulphate cuts were estimated for protein by measuring absorbance at 280 nm and also tested for the presence of activity towards the dipeptides Asp-Leu and Leu-Gly using the qualitative microassay screen. Fractions 25 to 35 and 48-58 from strain PN56 contained Asp-Leu hydrolysing activity as shown by the qualitative assay but only fractions 48-58 also contained Leu-Gly activity, indicating that these fractions contained peptidase B. Fractions 25-35 contained the peptidase E activity. Peptidase B containing fractions were assayed quantitatively for Leu-Gly hydrolysing activity and Peptidase E containing fractions for Asp-Leu activity (Fig. 13). Fractions 22 to 36 from strain PN31 contained peptidase E activity and these fractions were assayed for Asp-Leu hydrolysing activity (data not shown). Fractions containing peptidase activities were pooled and concentrated by ammonium sulphate precipitation.

Figure 13. Elution Of E.coli K12 Peptidase Activities From A DE52 Ion-Exchange Column



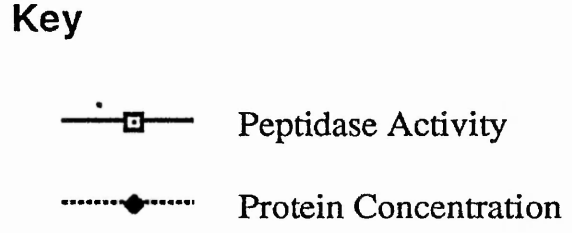
(1) Peptidase E Asp-Leu Hydrolysing Activity
(2) Peptidase B Leu-Gly Hydrolysing Activity

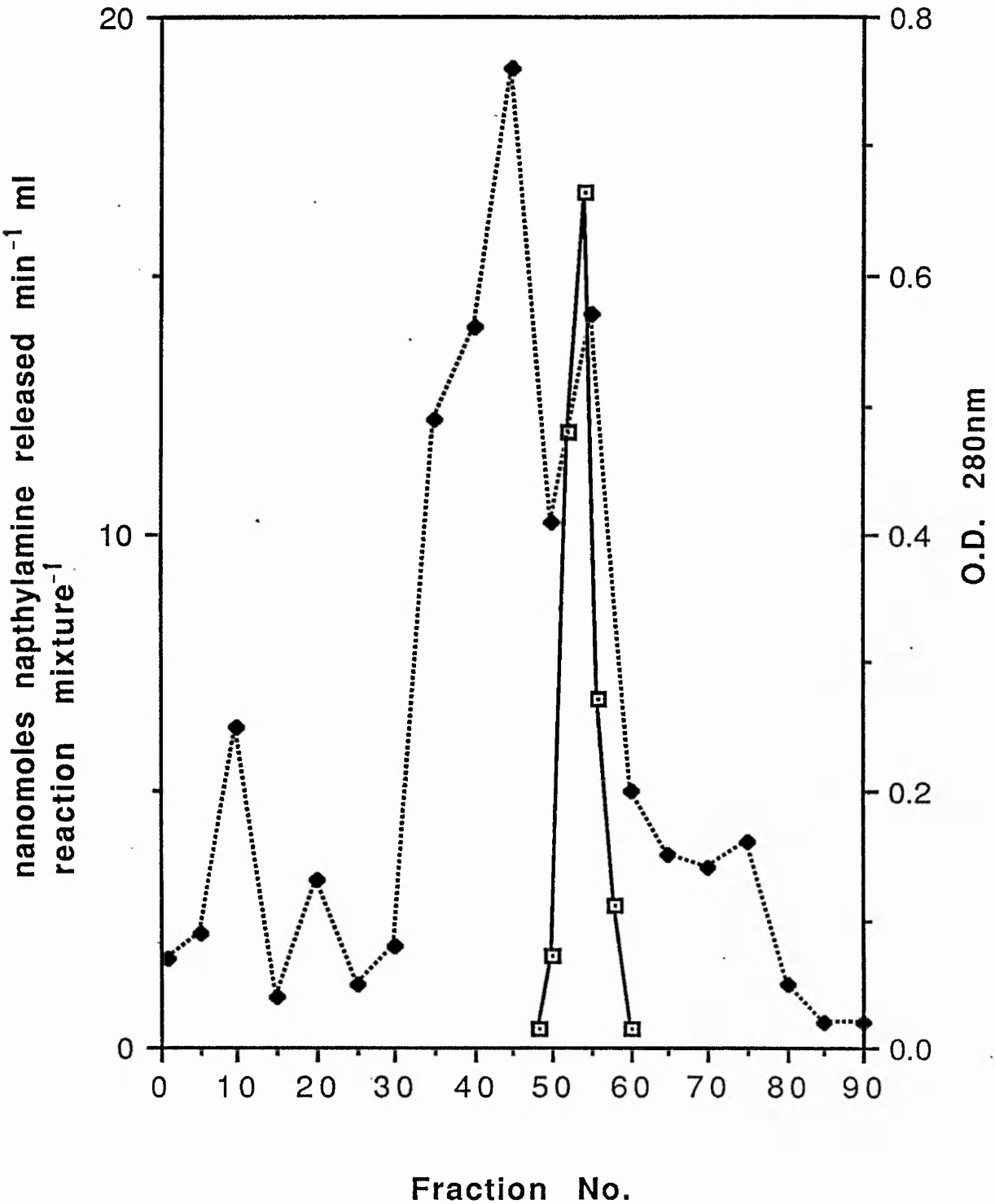

nanomoles leucine released min^{-1} ml reaction mixture^{-1}
O.D. 280nm
Fraction No.
(1)
(2)
0
2
4
6
8
10
12
14
0.0
0.2
0.4
0.6
0.8
1.0
0
10
20
30
40
50
60
70
80
90

Protein was resuspended in 0.05M Tris-HCl pH 7.5 then dialysed overnight at 4°C. The pooled and concentrated fractions were assayed for peptidase activity and protein. The pooled and concentrated Asp-Leu hydrolysing fractions from strain PN31 containing peptidase E gave a specific activity of 200 nmoles leucine released min^{-1} mg $protein^{-1}$ (0.2 units mg $protein^{-1}$) while the concentrated PN56 Leu-Gly hydrolysing fraction representing peptidase B gave a specific activity of 220 nmoles released min^{-1} mg $protein^{-1}$ (0.22 units mg $protein^{-1}$). These figures represent overall purification factors of 20- and 15-fold for peptidases B and E respectively. Maximum peptidase E activity was eluted at a volume of approximately 180 mls corresponding to a salt concentration of 0.18M. Maximal peptidase B activity eluted at approximately 306 mls corresponding to a salt concentration of 0.31M. Leu-Gly and Asp-Leu hydrolysing activities did not occur in the same fractions so the PN56 peptidase B and peptidase E activities could be separated by ion-exchange chromatography alone.

Fractions collected from ion-exchange of the A. calcoaceticus 40-60% ammonium sulphate cut, were assayed for protein and ANA hydrolysing activity (Fig. 14). Maximal ANA hydrolysing activity was eluted at a volume of 324 mls corresponding to a salt concentration of 0.32M. The fractions containing ANA hydrolysing activity were pooled and concentrated by ammonium sulphate precipitation with the

Figure 14. Elution Of A.calcoaceticus ANA Hydrolysing Peptidase Activity From A DE-52 Ion-Exchange Column



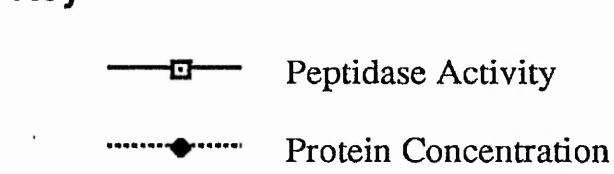

20
10
0
0.8
0.6
0.4
0.2
0.0
0 10 20 30 40 50 60 70 80 90
nanomoles napthylamine released min^{-1} ml reaction mixture^{-1}
O.D. 280nm
Fraction No.

protein precipitate being resuspended in 0.05M Tris-HCl pH 7.5 The specific activity of the pooled and concentrated fraction was determined at 400 nmoles napthylamine released min^{-1} mg $protein^{-1}$ (0.4 units mg $protein^{-1}$), representing an overall purification factor of 14.6.

4.2.2.4 Gel Filtration

Gel filtration was used as a means for further purification of peptidase enzymes and as a method for estimating peptidase molecular mass. Pooled and concentrated fractions from ion-exchange chromatography (1 ml) containing 5-10 mg protein and appropriate peptidase activities were applied to a 95 ml ($95ml = V_t - V_c$) column of Sephadex G200 (see Materials and Methods) and eluted with 0.05 M Tris-HCl pH 7.5 using a flow rate of 6 ml hr^{-1}. Fractions of the column effluent were collected and these were estimated for protein and for the appropriate peptidase activity using the qualitative microassay screen or by measuring ANA activity. G200 fractions derived from peptidase B and E ion-exchange fractions, which showed activity in the appropriate peptidase microassay screen were further assayed quantitatively for peptidase activity (Figs. 15 & 16). The peak elution volume (peak elution volume $\rightarrow V_e$) for peptidase E was determined at 44 mls and the corresponding V_e ($V_e - V_o$) for peptidase B was 12 mls. Marker proteins were applied to the G200 column and eluted under identical conditions to those employed for the partially purified peptidases. A calibration curve was constructed of $V_e - V_o / V_t - V_o$ against $\log_{10}$

Figure 15. Elution Of E.coli K12 Peptidase B (Leu-Gly Hydrolysing) Activity From A Sephadex G200 Gel Filtration Column



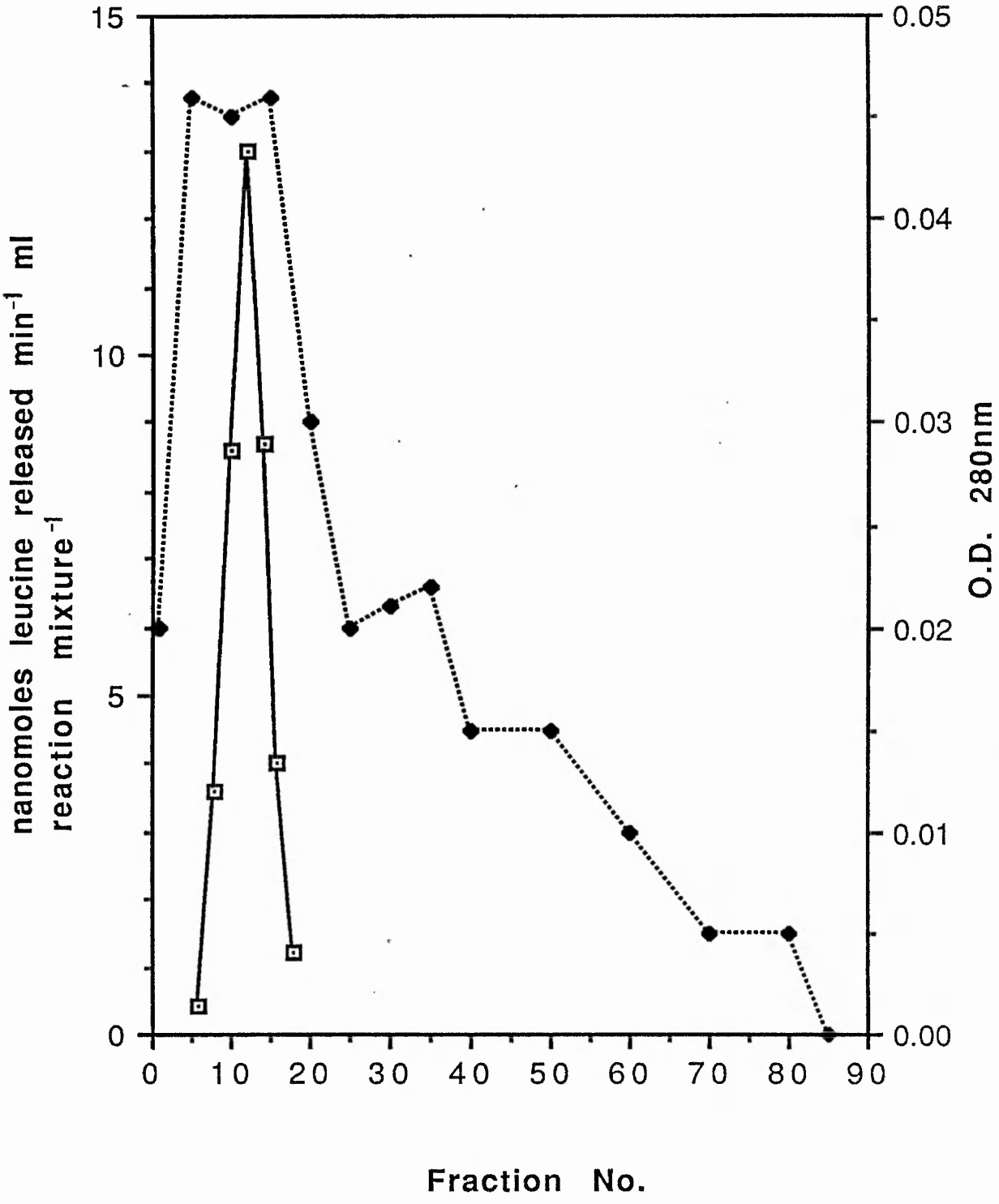
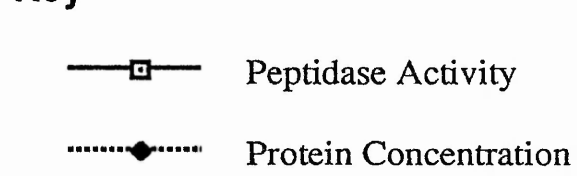
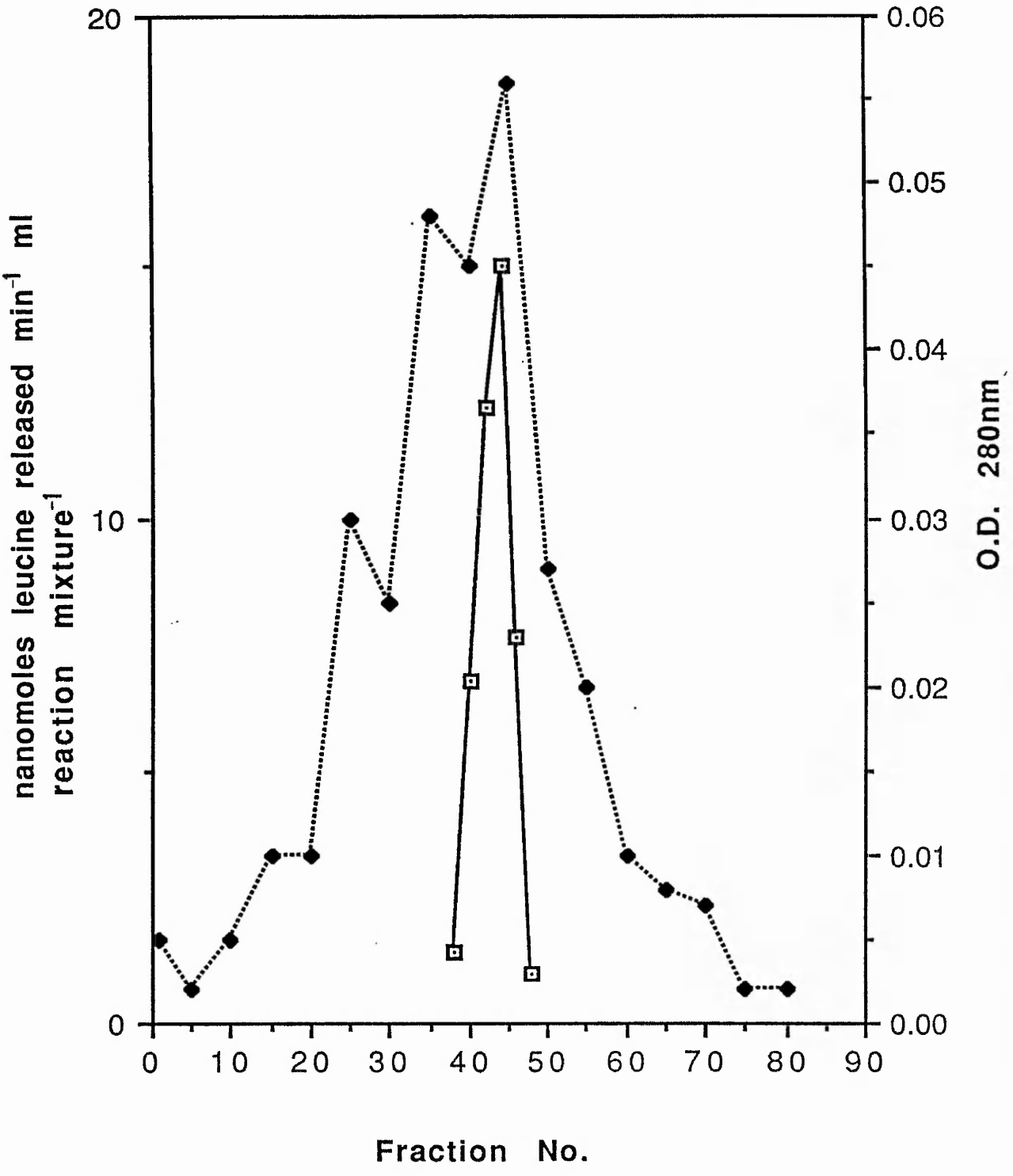

15
10
5
0
0.05
0.04
0.03
0.02
0.01
0.00
0 10 20 30 40 50 60 70 80 90
nanomoles leucine released min-1 ml reaction mixture-1
O.D. 280nm
Fraction No.

Figure 16. Elution Of E.coli K12 Peptidase E (Asp-Leu Hydrolysing Activity) From A Sephadex G200 Gel Filtration Column




20
10
0
0.06
0.05
0.04
0.03
0.02
0.01
0.00
0 10 20 30 40 50 60 70 80 90
nanomoles leucine released min-1 ml reaction mixture-1
O.D. 280nm
Fraction No.

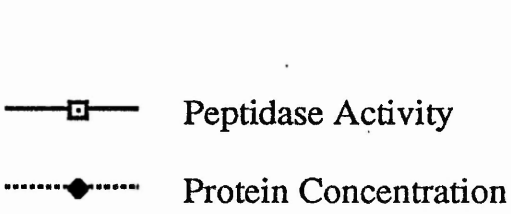
molecular mass using the data obtained for the marker proteins. From this curve, the molecular mass values for peptidases B and E were estimated at 230kd and 35kd respectively. Maximal aspartyl-β-napthylamide hydrolysing activity eluted at a volume of 6 mls (Fig. 17) corresponding to an M_r of = 480kd.

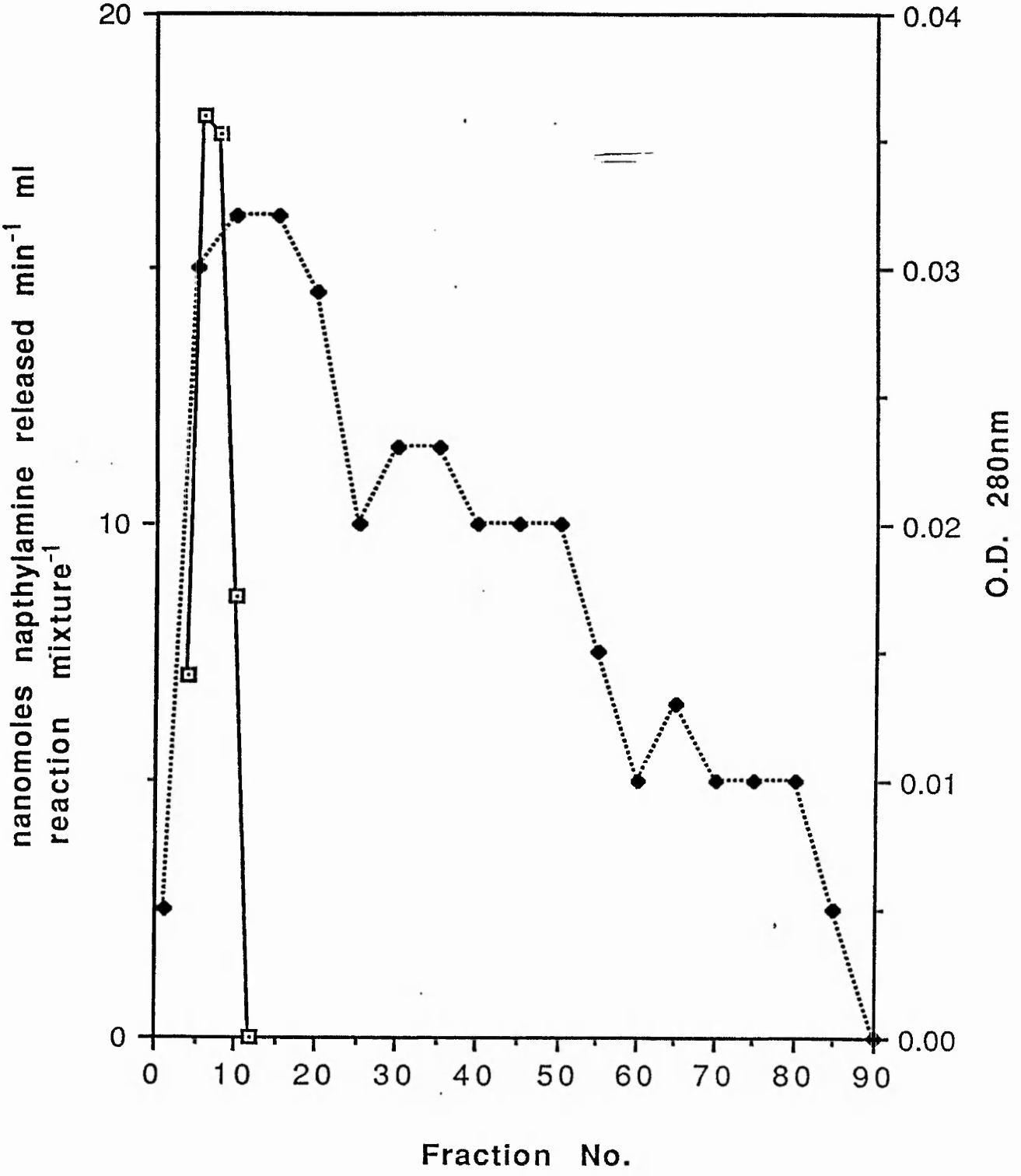
Fractions from gel filtration were pooled and concentrated by ammonium sulphate precipitation and after dialysis peptidase activities were determined. The purified PN56 peptidase B and PN31 peptidase E fractions gave specific activities of 1,000 and 1,300 nmoles leucine released min^{-1} mg $protein^{-1}$ from their respective substrates (1.0 and 1.3 units mg $protein^{-1}$) representing purification factors of 91 and 100-fold respectively. ANA hydrolysing activity was determined at 3,500 nmoles napthylamine released min^{-1} mg $protein^{-1}$ (3.5 units mg $protein^{-1}$) representing a purification factor of 128.

Overall yields from the partial purification procedure were determined as 3.8% and 4.5% for the PN56 peptidase B and peptidase E respectively, and 4.3% for the A. calcoaceticus ANA hydrolysing peptidase.

These 3 partially purified peptidase containing fractions were used in quantitative assays aimed at analysing the factors affecting enzyme activity, relative substrate specificities and other peptidase characteristics.

Figure 17. Elution Of A.calcoaceticus ANA Hydrolysing Peptidase Activity From A Sephadex G200 Gel Filtration Column



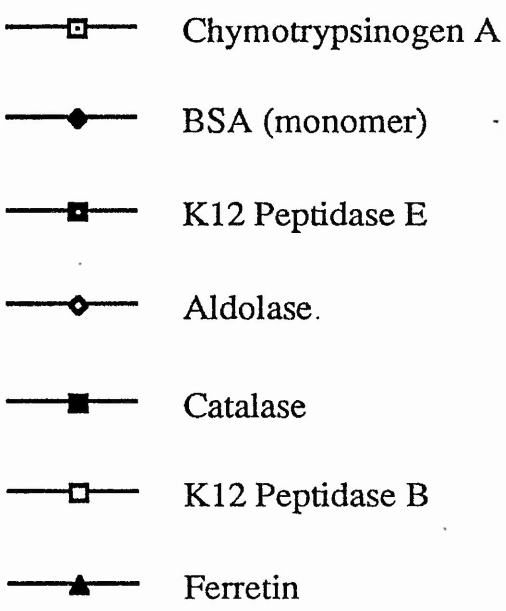

20
10
0
0.04
0.03
0.02
0.01
0.00
0 10 20 30 40 50 60 70 80 90
Fraction No.
nanomoles napthylamine released min-1 ml reaction mixture-1
O.D. 280nm

4.2.3 Characterisation of the *E. coli* K12 Aspartyl Peptidase Enzymes

4.2.3.1 Confirmation of Molecular Mass values (M_r) by Polyacrylamide Gel Electrophoresis on Non-denaturing Gels

50 µl samples of PN56 crude cell extract were applied to non-denaturing polyacrylamide disc gels with acrylamide concentrations ranging from 4-16%. After electrophoresis gel tubes were removed and stained for peptidase activity towards Asp-Leu. Mobilities of the bands of peptidase activity were recorded for each peptidase on each different concentration of acrylamide and graphs plotted of mobility versus % acrylamide (Fig. 18). Mobilities of molecular mass markers on different concentrations of acrylamide were also determined, plotted on the same graph and the slopes of the lines thereby produced were themselves plotted against marker molecular mass. The plot of these retardation coefficients ($-K_R$) against molecular mass gave a standard curve (Fig. 19); reading off the retardation coefficient values for the peptidases B and E gave molecular mass values of 265kd and 38kd respectively. These values correspond closely with the values of M_r for the 2 enzymes as determined from gel filtration. Errors arising at $\pm$ 4% have been reported for molecular mass values determined using the non-denaturing PAGE method (Hedrik & Smith, 1968). These workers constructed accurate standard curves by use of numerous molecular mass standards. In the experiments

Figure 18. Mobilities Of E.coli K12 Peptidases And Molecular Mass Markers After Electrophoresis On Differing Concentrations Of Acrylamide



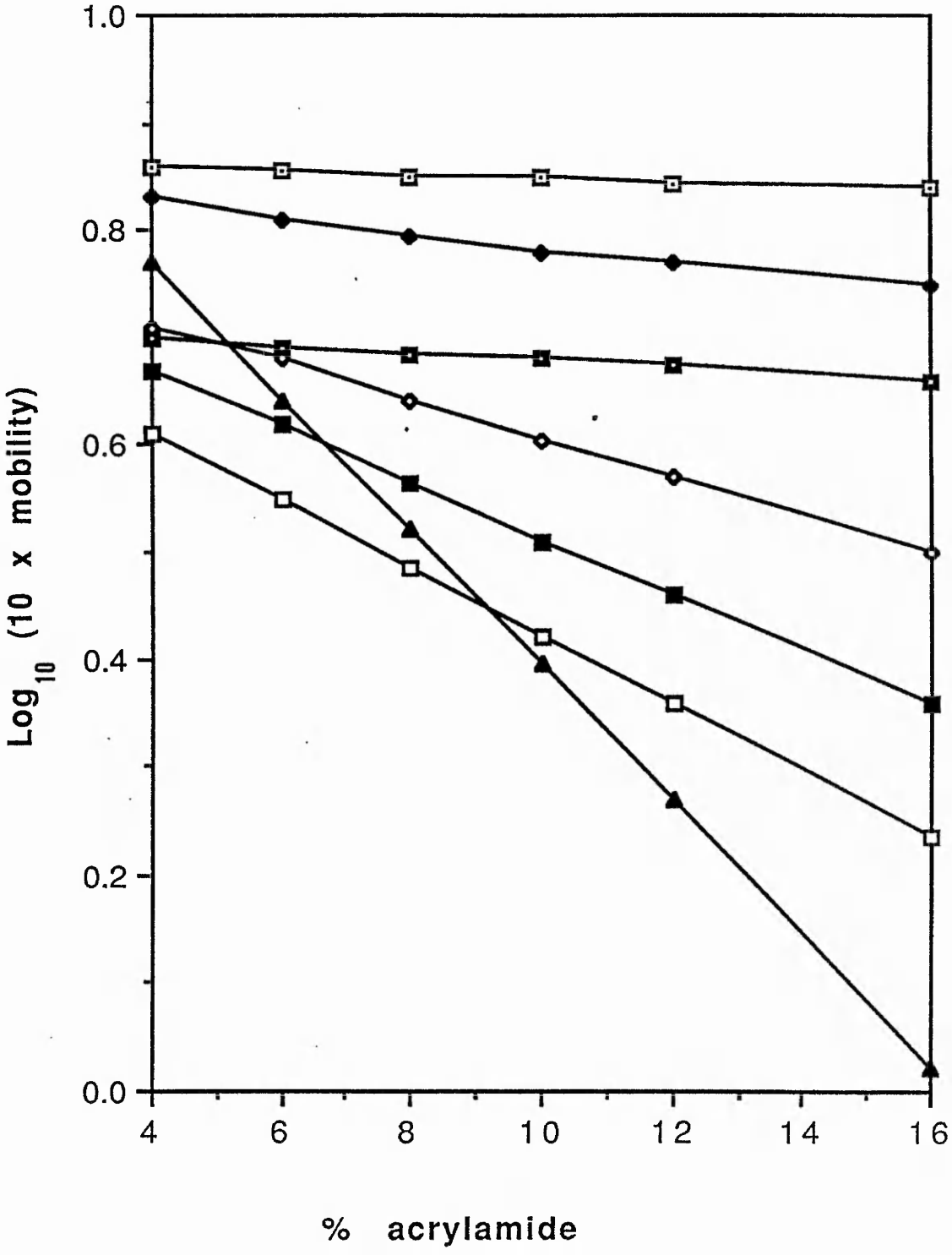
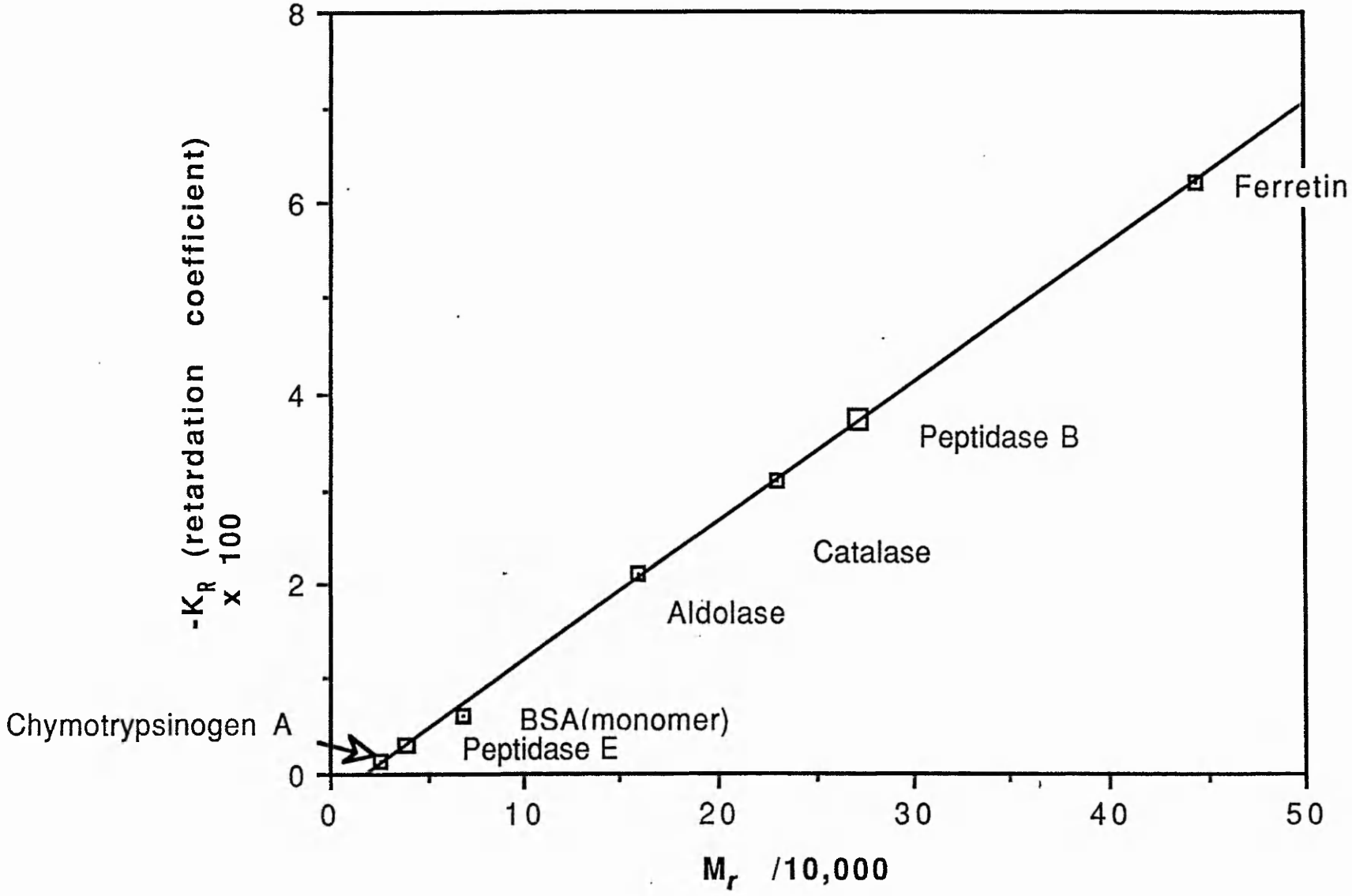

1.0
0.8
0.6
0.4
0.2
0.0
Log10 (10 x mobility)
4
6
8
10
12
14
16
% acrylamide

Figure 19. Determination Of Molecular Mass values For E.coli K12 Aspartyl Peptide Hydrolysing Enzymes Using PAGE Retardation Coefficients



reported here fewer standards were used and a higher degree of error would be expected. Such error most likely accounts for the molecular mass differences obtained by the 2 methods used. The presence of single peptidase B and peptidase E activity bands from crude extracts at each concentration acrylamide gel confirmed that the peptidase B and E activity bands observed on initial 7.5% gel assays represented single enzyme activities.

4.2.3.2 Substrate Specificities of *E. coli* K12 Peptidases B and E

Initial investigations on the substrate specificity of the aspartyl-peptidase hydrolysing enzymes B and E were carried out using the qualitative peptidase stain, after separation of crude cell extracts from peptidase containing strains on 7.5% non-denaturing polyacrylamide gels. Separated crude cell extracts were stained for activity towards a number of different peptide substrates including N-terminal and C-terminal aspartyl peptides as well as leucyl, glutamyl and asparaginyl peptides. The presence or absence of activity bands at mobilities previously ascribed to the 2 peptidases was noted and taken to represent the occurrence or non-occurrence of peptidase catalysed hydrolysis (Table 11).

Only N-terminal l-α-aspartyl peptides proved to be hydrolysed by the activity band (mobility 0.5) representing

Table 11. Substrates producing Activity Bands representing the different *E. coli* K12 Peptidases on PAGE assay staining

Peptide	Peptidase B	Peptidase E
Asp-Leu	+	+
Asp-Phe	+	+
Asp-PheOMe	+	+
Asp-Tyr	+	+
Leu-Gly	+	-
Leu-Phe	+	-
Leu-Leu	+	-
Phe-Asp	-	-
Phe-Leu	+	-
Phe-Phe	+	-
Glu-Phe	-	-
Glu-Leu	-	-
Asn-Phe	-	-

peptidase E. The peptidase B activity band (mobility 0.3) was observed with both leucyl, phenylalanyl and N-terminal aspartyl peptides as assay substrates. N-terminal glutamyl peptides or asparaginyl peptides were not hydrolysed by either aspartyl peptidase, nor were the C-terminal aspartyl-peptide Phe-Asp and the chromophoric aspartyl-β -napthylamide. As Phe-Asp was shown to be a growth substrate for peptidase deficient strains (Chapter 3) this indicates the presence of at least one peptidase activity which is not detectable using the gel assay system.

Partially purified peptidases B and E from ammonium sulphate fractionnation, ion-exchange chromatography and gel filtration were assayed for their activity towards L-α -aspartyl and other peptides, by incubation of 0.005 units of each enzyme activity with substrates followed by fluorescence analysis of OPA derivatised products after their separation by HPLC. Substrate specificities for each peptidase were determined relative to hydrolysis rates with the substrate Asp-Leu (Table 12). Asp-Leu was hydrolysed by the partially purified peptidase E at a rate of 1.3 μmoles min^{-1} mg $protein^{-1}$ whereas the Asp-Leu hydrolysis rate for peptidase B was determined at 0.68 μmoles min^{-1} mg $protein^{-1}$. All aspartyl peptides hydrolysed by the peptidase E enzyme were also hydrolysed by peptidase B in the quantitative assays. N-terminal L-α-aspartyl peptides with small hydrophobic C-terminal residues appeared to be

Table 12 Substrate Specificities of Partially Purified *E. coli* K12 Aspartyl Peptide Hydrolysing Enzymes

Substrates	Rates of Hydrolysis[a]	
	Peptidase B	Peptidase E
Asp-Leu	100	100
Asp-Ala	142	165
Asp-Phe	45	60
Asp-Val	160	105
Asp-Tyr	65	83
Asp-PheOMe	ND	27
Asp-Lys	83	140
Asp-Gly	162	54
Asp-Glu	43	57
Leu-Gly	144	0
Asp-β-napthylamide	0	0
CBZ-Asp	0	0
Phe-Asp	0	0

[a] Substrate specificities are expressed relative to the rates of Asp-Leu hydrolysis for each peptidase (see text).

efficient substrates for both peptidases with aromatic C-terminal and acid C-terminal residue aspartyl peptides being poorly hydrolysed. The dipeptides Asp-Gly, Asp-Val and Asp-Lys show significantly different degrees of hydrolysis between the 2 enzymes. CBZ-Asp, Asp-β -napthylamide and Phe-Asp were not hydrolysed by either peptidase.

Sonicated crude cell extracts from strain PN31 were also used in quantitative substrate specificity assays and generated a substrate specificity profile identical with that obtained for partially purified peptidase E.

4.2.3.3 Effects of pH on Aspartyl-Peptide Hydrolysis by Peptidases B and E

The activity of the partially-purified aspartyl-peptide hydrolysing peptidases towards the dipeptide Asp-Leu was assayed over the pH range 5.0-10.0 in Tris-HCl buffer (Figs. 20 and 21). Maximum hydrolysis rates were achieved for both peptidases at pH 7.8, with maximum activity for peptidase E catalysed hydrolysis determined at 1.35 μmoles min^{-1} mg $protein^{-1}$ and for peptidase B at 0.72 μmoles min^{-1} mg $protein^{-1}$. Significant peptidase E activity was observed at around pH 10 whereas peptidase B activity was negligible at this pH. Subsequent assays on peptidases were carried out at pH 7.5 as this pH gave high levels of activity and had been used for previous qualitative and quantitative assays

Figure 20. pH/Activity Profile For E.coli K12 Peptidase B Catalysed Hydrolysis Of Asp-Leu

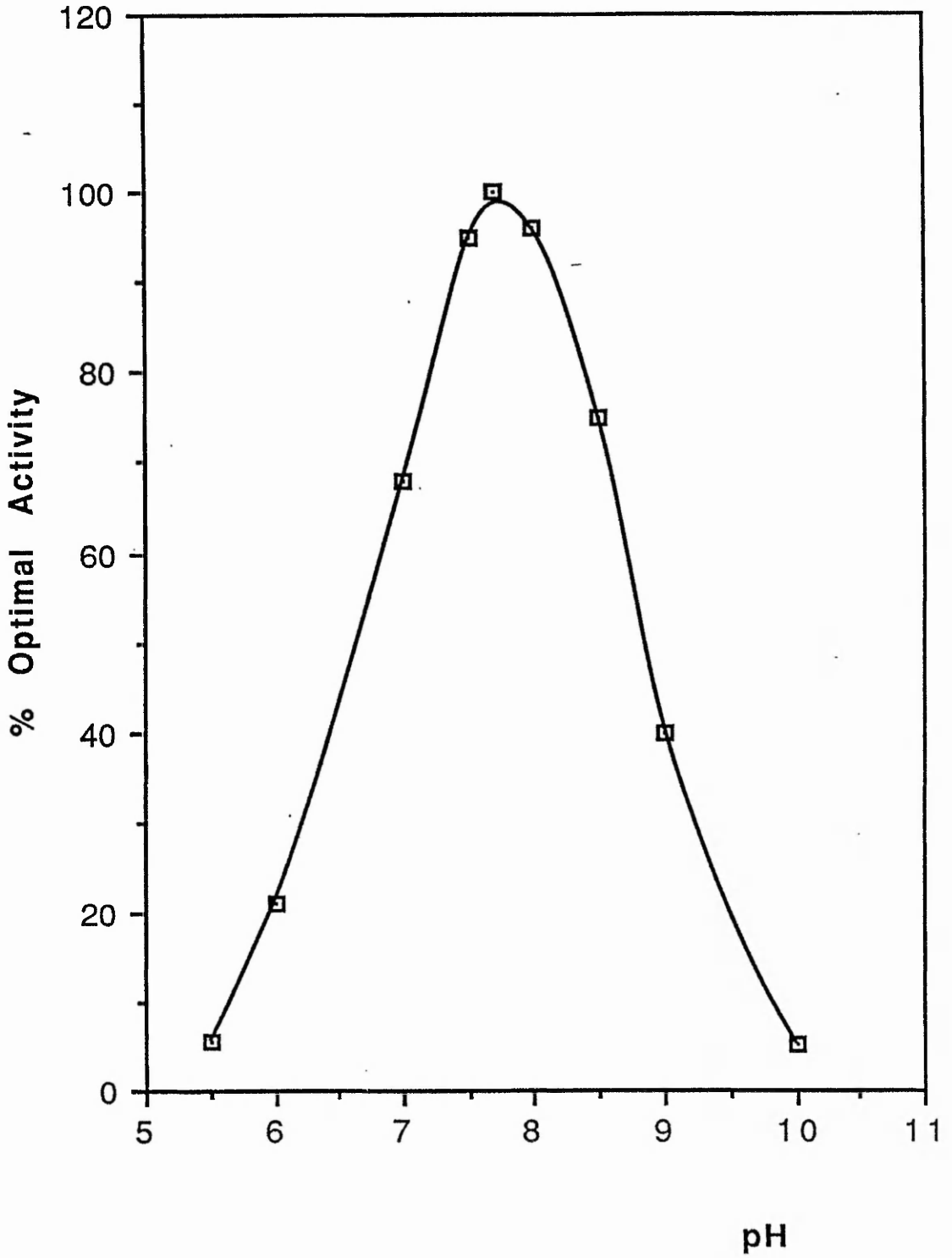
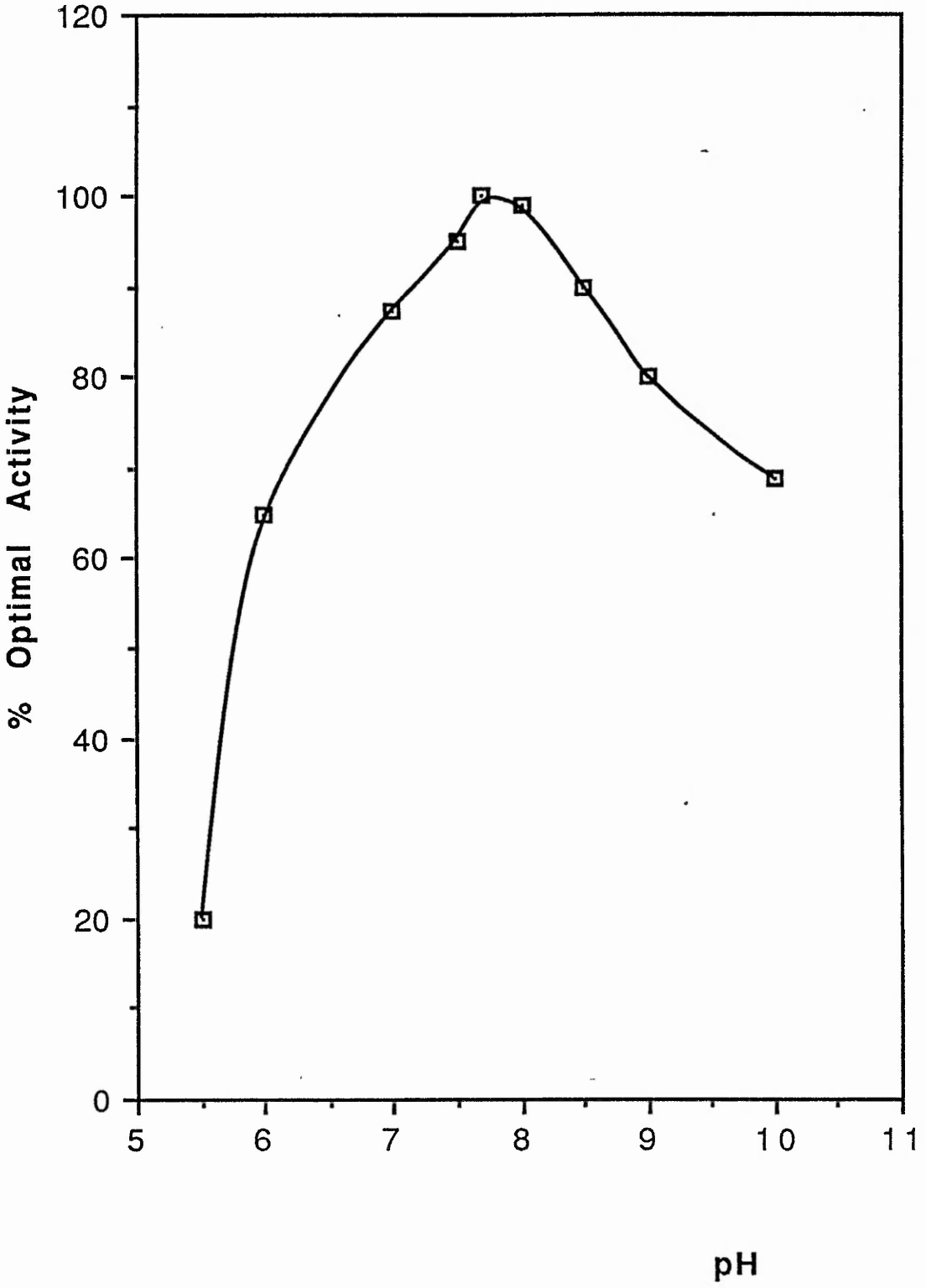


Figure 21. pH/Activity Profile For E.coli K12 Peptidase E Catalysed Hydrolysis Of Asp-Leu



during preliminary analysis of crude cell extracts and during purification.

4.2.3.4 Effects of Metal Ions and Chelating Agents on Peptidase Activities

For analysis of metal ion effects using the PAGE activity stain, sonicated crude cell extracts were separated on gels which were then soaked for 30 minutes in 1mM solutions of metal ions. Gels were stained for activity towards dipeptides using peptidase staining mixture minus Mn^{2+}. The Asp-Leu hydrolysing activity band representing peptidase E appeared unaffected in its activity by $CoCl_2$, $ZnCl_2$, $MnCl_2$ and $FeCl_3$ or by the metal ion chelating agent EDTA. Peptidase B activity towards both Asp-Leu and Leu-Gly appeared to be stimulated by the presence of Mn^{2+} as indicated by a broadening of the peptidase B activity band. This activity band was completely lost on preincubation of gels in Zn^{2+} with both peptide substrates. Preincubation of gels in 1mM EDTA also resulted in the complete loss of the peptidase B activity band. This data confirms the previous studies (Hermsdorf & Simmonds, 1980) which suggested that peptidase B was an Mn^{2+} dependent metallopeptidase, but provides no clues as to the factors which are involved in peptidase E activity.

4.2.3.5 Stability studies on Peptidases B and E

The stability of partially purified peptidase

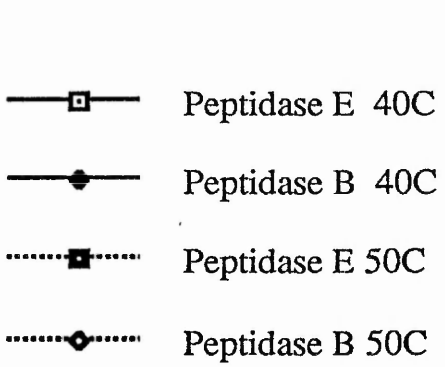
activities were examined after 24 hrs storage in 0.1 M Tris-HCl pH 7.5 at 4°C and -20°C. Peptidase activity towards Asp-Leu were measured before and after exposure at a given temperature. Peptidase E was found to be stable at both temperatures with no detectable loss of activity over the time period of incubation. Storage of peptidase B at 4°C produced a 5% loss of Asp-Leu hydrolysing activity over 24 hrs and ~~a~~ 7% loss of degrading activity was observed on storage at -20°C.

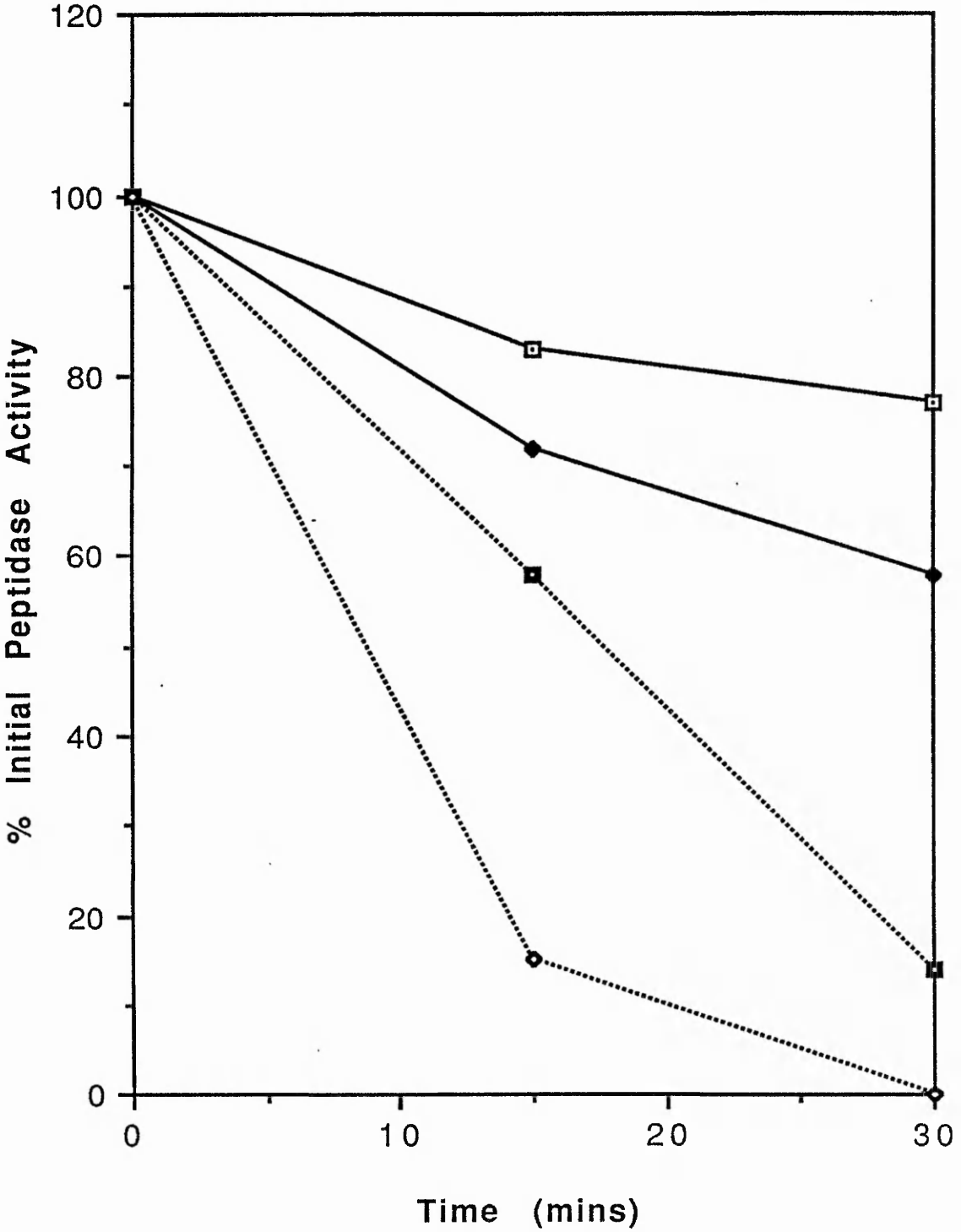
More detailed studies on the effect of temperature on the stability of the 2 peptidase activities were carried out by exposing samples of purified peptidases in 0.1M Tris-HCl pH 7.5, to either 40°C or 50°C for 30 minutes during which time samples were withdrawn, cooled and assayed for Asp-Leu hydrolysing activity (Fig. 22). Exposure of both peptidases B and E to 40°C resulted in a decrease in Asp-Leu degrading activity which was more marked for peptidase B. Rapid loss of activity was observed with both peptidases at 50°C.

4.2.3.6 Effects of Inhibitors on Aspartyl-Peptide Hydrolysis by Peptidases B and E

Experiments using the gel activity stain demonstrated that peptidase B catalysed hydrolysis of Asp-Leu was strongly inhibited by the metal ion chelating agent EDTA when this reagent was present at a concentration of 1mM. At this same concentration the reagent had no observable effect

Figure 22. Heat Stability Of E.coli K12 Peptidases B and E On Incubation At 40C And 50C




120
100
80
60
40
20
0
% Initial Peptidase Activity
0
10
20
30
Time (mins)

on peptidase E activity. Quantitative studies on the effects of EDTA and other potential enzyme inhibitors on the Asp-Leu hydrolysing activities of both peptidases B and E were carried out by incubating partially purified enzymes and substrate in the presence of 1mM inhibitor (Table 13). Somewhat surprisingly the activity of peptidase E appeared to be stimulated by the presence of EDTA whereas the sulphydryl enzyme inhibitor PCMB (para~~poly~~mercuribenzoate) and the serine protease inhibitor PMSF (phenylmethylsulphonyl-fluoride) had little effect on its activity. Peptidase B on the other hand was substantially inhibited in its Asp-Leu hydrolytic activity by EDTA and only slightly affected by the other enzyme inhibitors to a degree which might reflect the metal-ion chelating properties of these reagents. Neither peptidase E or B appear to be serine protease or sulphydryl enzymes.

4.2.4 Characterisation of the ANA-Hydrolysing Peptidase from A. calcoaceticus

4.2.4.1 Substrate Specificity Studies

Analysis of resultant peptidase activity bands after separation and activity staining of A. calcoaceticus crude extracts on polyacrylamide gels showed that the A. calcoaceticus ANA hydrolysing peptidase had the same mobility (0.02) as an Asp-Leu and Asp-Phe hydrolysing activity band. This same activity band appeared after activity staining towards a range of N-terminal L-α-aspartyl

Table 13. Effects of Enzyme Inhibitors on Partially Purified *E. coli* PN56 Aspartyl-Peptide Hydrolysing Activities

Inhibitor (1mM)	% Optimum Activity	
	Peptidase B	Peptidase E
None	100	100
EDTA	2	175
PMSF	50	80
PCMB	75	100

peptides but did not appear with leucyl, glutamyl, phenylalanyl, asparaginyl peptides or the C-terminal dipeptide Phe-Asp as assay substrates (Table 14).

Quantitative assays using partially purified preparations of the ANA hydrolysing peptidase using the HPLC assay procedure with peptides present at 3mM confirmed that this peptidase could hydrolyse a range of L-α-aspartyl peptides (Table 15) and hence was the same enzyme that hydrolysed these peptides in the gel stained crude extracts. The ANA peptidase showed highest levels of hydrolysis with L-α-aspartyl peptides containing aromatic or hydrophobic C-terminal residues. No detectable hydrolysis was observed with Phe-Asp, CBZ-Asp or the dipeptide Leu-Gly as assay substrates. Hydrolysis was determined relative to hydrolysis rates with the substrate Asp-Leu which was hydrolysed at a rate of 2.3 units mg protein^{-1}.

4.2.4.2 Effects of pH on the ANA Hydrolysing Activity

The pH optimum for the hydrolysis of aspartyl-β -napthylamide by the ANA hydrolysing peptidase was determined by assaying partially purified peptidase for ANA hydrolysis in Tris-buffers ranging from pH 5-10.

Optimum rates of activity were achieved at pH 7.8 reaching a maximum of 4.2 units mg protein^{-1} (Fig. 23). Substantial hydrolytic activity was retained at a pH value

Table 14. Substrates producing Activity Bands for the ANA Hydrolysing Peptidase of A. calcoaceticus on PAGE assay staining

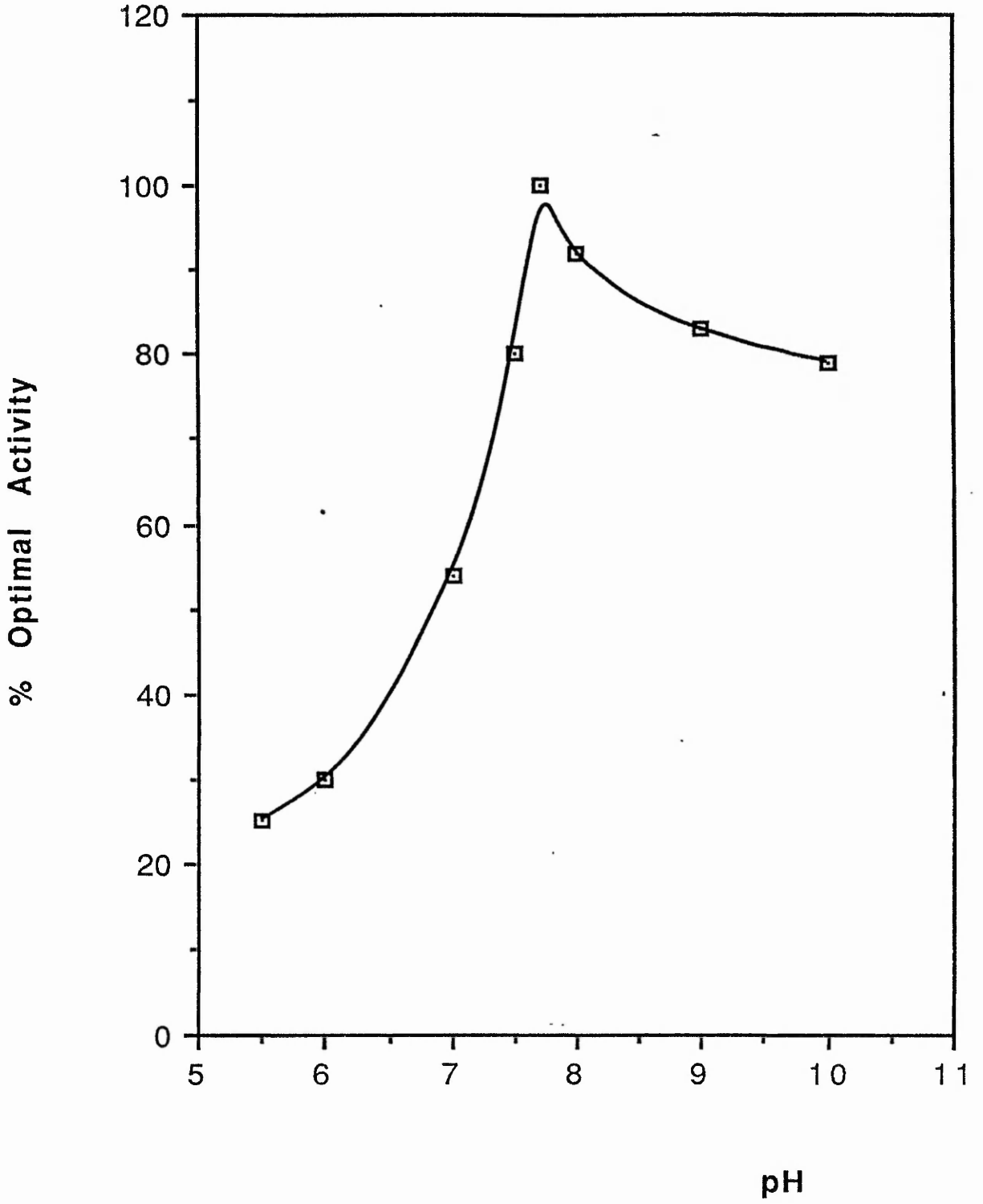
Peptide	ANA Peptidase
Asp-Leu	+
Asp-Phe	+
ANA	+
Asp-PheOMe	+
Asp-Tyr	+
Leu-Gly	-
Leu-Phe	-
Leu-Leu	-
Phe-Asp	-
Phe-Leu	-
Phe-Phe	-
Glu-Phe	-
Glu-Leu	-
Asn-Phe	-

Table 15. Substrate specificities of partially purified *A. calcoaceticus* ANA Hydrolysing Peptidase

Peptide	Rate of Hydrolysis[a]
Asp-Tyr	206
Asp-PheOMe	205
Asp-Phe	205
Asp-Ala	198
Asp-Val	151
Asp-Lys	142
Asp-Leu	100
Asp-Gly	60
Asp-Glu	39
CBZ-Asp	0
Phe-Asp	0
Leu-Gly	0

a Substrate specificities are expressed relative to the rate of Asp-Leu hydrolysis (see text).

Figure 23. pH/Activity Profile For A.calcoaceticus ANA Hydrolysing Peptidase Activity



up to 10.0 but was rapidly lost below pH 7.7. Assays were carried outsubsequently at pH 7.5 as this level of hydrogen ion concentration gave substantial levels of ANA hydrolysis while being consistent with the pH of buffers used in other enzyme assays and used in both gel assays and peptidase purification.

4.2.4.3 Effect of Temperature on ANA Hydrolysis

Optimum rates for peptidase catalysed hydrolysis of ANA by partially purified ANA peptidase were achieved at 37°C (3.5 units mg protein^{-1}). More than 50% of ANA hydrolytic activity was retained over the range 20°C-37°C whereas above 37°C activity was rapidly lost and no enzyme activity at all was observed at 50°C (Fig. 24). Thus this enzyme appears to operate most efficiently at the 37°C optimum growth temperature of A. calcoaceticus.

4.2.4.4 Effects of Metal Ions on ANA Hydrolysing Activity

Samples of the partially purified ANA Peptidase were assyaed quantitatively for ANA hydrolysis in the presence of 1mM salts. The metal ions Mn^{2+} and Ni^{2+} reduced levels of peptidase activity by over 60% giving strong inhibition; Ca^{2+}, Zn^{2+} and Co^{2+} inhibited peptidase activity to a lesser extent while Mg^{2+} and Na^{+} had no significant effect on enzyme activity (Table 16). The presence of 1mM EDTA resulted in the loss of over 90% of peptidase activity. The effects of EDTA and the observed metal inhibition suggested

Figure 24. Temperature/Activity Profile For The A.calcoaceticus ANA Hydrolysing Peptidase Activity

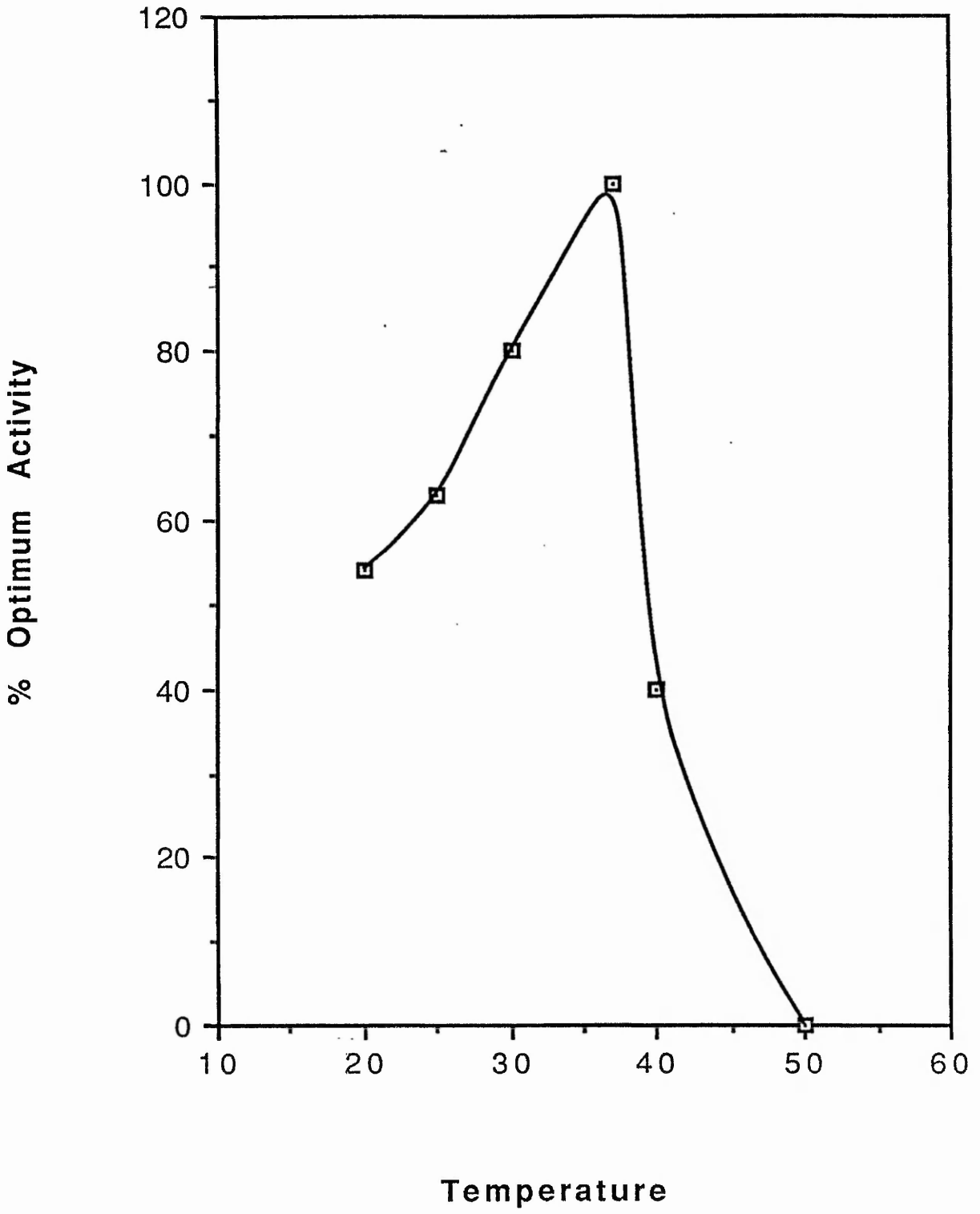


Table 16. Effects of Metal Ions on ANA Hydrolysing Activity of Partially Purified A. calcoaceticus peptidase

Salt (1mM)	% Optimal ANA Hydrolysing Activity
-	100
NaCl	100
$MgCl_2$	98
$ZnCl_2$	81
$CaCl_2$	81
$CoCl_2$	68
$MnCl_2$	34
$MnSO_4$	32
$NiCl_2$	18
EDTA	6

that the ana degrading enzyme is a metallopeptidase. Dialysis of crude extract at 4°C overnight against 2mM EDTA resulted in almost total loss of enzyme activity. Addition of Mg^{2+} ions restored the enzyme activity to original levels suggesting that the ANA hydrolysing enzyme may be an Mg^{2+} dependent metallopeptidase. The level of inhibition by Mn^{2+} ions was not altered by altering the form of salt in which the metal ion was supplied. The form of anion appears from this result to have little effect on the enzyme activity levels.

4.2.4.5 Stability studies

Sample of partially purified ANA peptidase were incubated for 24 hrs at different temperatures and samples were assayed for ANA hydrolysis before and after incubation. 2.5% of ANA hydrolysing activity was lost on incubation at 4°C and 5% after incubation at -20°C. Only 5% of activity remained after incubation at 37°C and all activity disappeared after 24 hrs incubation at 45°C and 50°. The ANA hydrolysing peptidase appears from this data to be unstable at the optimum 37°C physiological growth temperature of A. calcoaceticus.

4.2.4.6 Inhibition Studies

Samples of partially purified peptidase were assayed for ANA hydrolysing activity in the presence of potential enzyme inhibitors at a concentration of 1mM. The most

active enzyme inhibitor was the metal-ion chelator 1,10 phenanthroline where only 4% of optimal activity was observed. The other metal-ion chelators EDTA, α,α-dipyridyl and bathophenanthroline sulphonate also induced high levels of inhibition. The sulphydryl enzyme inhibitors PCMB and iodoacetamide had little effect on enzyme activity nor did the serine protease inhibitor PMSF affect activity (Table 17). The levels of inhibition observed for the sulphydryl inhibitors were too low to suggest the involvement of sulphydryl enzymes in the enzyme active site. The action of the metal-ion chelators in producing high levels of enzyme inhibition reinforces the metal ion effect data which indicates that the ANA hydrolysing enzyme is a metallopeptidase.

4.3 Discussion

In the work detailed in this chapter, the E. coli K12 aspartyl-peptide hydrolysing enzymes peptidase B and E were partially purified from crude cell extracts. Such purification used a 3 stage process as described and gave respectively 91 fold and 100 fold purification. Samples of peptidase containing fractions from this purification were used in quantitative assays as these fractions contained enough activity to enable a large number of assays to be carried out. The ANA hydrolysing enzyme from A. calcoaceticus was similarly purified 128-fold using the same procedures.

Table 17. Effects of Inhibitors on ANA Hydrolysing Activity of Partially Purified A. calcoaceticus peptidase

Inhibitor (1mM)	% Optimal ANA Hydrolsying Activity
-	100
1,10 phenanthroline	4
EDTA	6
Bathophenanthroline	34
Dipyridyl	47
PMSF	100
Amytal	100
Sodium Azide	96
PCMB	94
Iodoacetamide	92

The data obtained from the enzyme characterisation experiment reveals both physical and biochemical differences in the properties of the aspartyl-peptidases studied. The 2 E. coli K12 aspartyl-peptide hydrolysing enzymes, designated peptidases B and E, gave respectively molecular mass values of approximately 230kd and 35kd. The M_r value for peptidase B corresponds closely with previously determined values (Hermsdorf, 1978; Hermsdorf & Simmonds, 1980). Both of these E. coli K12 peptidases possess the ability to hydrolyse a range of different N-terminal L-α-aspartyl dipeptides although the degree of specificity towards each different aspartyl-peptide varies between the 2 enzymes. Both peptidases appeared to have a preference for N-terminal aspartyl peptide substrates with small hydrophobic C-terminal residues. Whereas E. coli K12 peptidase E was restricted in its substrate specificity to aspartyl-peptide substrates, peptidase B would hydrolyse both aspartyl peptides and N-terminal or C-terminal leucyl dipeptides. Neither enzyme would hydrolyse the C-terminal aspartyl peptide Phe-Asp, N-terminal asparaginyl or glutamyl peptides, the amino acid napthylamide ANA, or the aspartate derivative CBZ-Asp. Specificity of these peptidases towards aspartyl-tripeptides or D-aspartyl peptides was not tested due to either the non-availability or expense of these test substrates. These overlapping substrate specificities and differences in substrate specificity may reflect functional differences in the cellular roles of these peptidases but

may also relate to a cellular requirement for degradation of a broad range of aspartyl and other peptides, a function requiring more than one peptidase.

The 2 E. coli K12 aspartyl-peptidases showed the same pH optimum (pH 7.8) for maximal hydrolysis of the Asp-Leu dipeptide. The pH range of each enzyme activity varied with both peptidases B and E losing activity rapidly below pH 7.5 but substantial peptidase E remaining at pH 10 in contrast to peptidase B where over 90% of Asp-Leu hydrolytic activity was lost at this pH. Again this may reflect some functional difference in the cellular roles of these 2 peptidases.

The E. coli K12 peptidase E appears to be more stable than the peptidase B enzyme on storage at 4°C and -20°C as well as on exposure to higher temperatures. Contrary to peptidase B however, this enzyme was unaffected in activity by metal ions and was not inhibited by EDTA. Use of an alternative metal-ion chelating agent such as O-phenanthroline in quantitative assays might have demonstrated a metal-ion requirement for E. coli K12 peptidase E. All E. coli K12 peptidases previously described have been demonstrated to have a metal-ion requirement for activity and such a requirement for peptidase E could explain the observed enhancement of activity observed on incubation of this enzyme with EDTA. A possible explanation for this phenomenom would involve

chelation of trace metal ions which could compete with a metal-ion tightly bound to the enzyme activity site.

Peptidase B was confirmed as a metallopeptidase due to the inhibition of this activity by Zn^{2+} and EDTA as well as the observed enhancement of its activity in the presence of Mn^{2+} confirming the observations of other workers (Hermsdorf & Simmonds 1980). Such metal ion effects were observed both with aspartyl and leucyl peptides which may point to a single active site for hydrolysis of both types of peptide. It remains possible however that separate binding sites exist on this large peptidase for different types of peptide substrate. This situation could be resolved by competition experiments. The observed effects do suggest however similar mechanisms of hydrolysis for both types of dipeptide substrate.

The observation of a bell-shaped pH versus enzyme activity graph may indicate the involvement of an acid:base mechanism of catalysis for aspartyl-peptide hydrolysis by E. coli K12 peptidase B. In the zinc protease carboxypeptidase A, a bell-shaped pH activity curve with optimum activity at pH 7.5 is thought to be due to the involvement of the basic form of a catalytic glutamate residue $pK_a 6$ and the acidic form of an as yet unassigned group $pK_a 9.1$ in the free enzyme (Ferscht A.J. 1987). Such a situation may pertain with peptidase B. Neither E. coli K12

aspartyl-peptidase enzyme appeared to be significantly inhibited by serine protease or sulphydryl enzyme inhibitors.

It was proposed in Chapter 3 that additional aspartyl-peptide hydrolysing activities might be present in E. coli K12 as well as peptidases B and E. No such additional activities were identified during the purification protocol, nor was any activity observed corresponding to the previously described aminopeptidase L.

The ANA hydrolysing peptidase from A. calcoaceticus was found to have a molecular mass of approximately 480kd. Like the E. coli K12 peptidases B and E, this ANA hydrolysing enzyme hydrolysed a range of N-terminal aspartyl peptides but would not hydrolyse C-terminal peptides. Like the E. coli K12 peptidase E it would however only hydrolyse aspartyl peptides but not the leucyl, asparaginyl, glutamyl or phenylalanyl peptides tested as substrates. Moreover the ANA hydrolysing peptidase had a higher affinity for aspartyl-dipeptides carrying a C-terminal residue with an aromatic side chain, revealing another difference between this enzyme and the E. coli K12 peptidases, which showed no special affinity for this type of substrate.

The ANA hydrolysing enzyme was also found to be a metallopeptidase inhibited by EDTA and other metal ion

inhibitors while other serine protease and sulphydryl enzyme inhibitors had little effect on activity. The enzyme showed no unusual stabilities and gave optimum hydrolytic activity like the E. coli K12 peptidase at pH 7.8. Like E. coli K12 peptidase E the ANA hydrolysing peptidase retained substantial activity at high pH.

A major similarity between all of the peptidases isolated was their specificity for N-terminal but not C-terminal L-α-aspartyl-peptides. The presence and therefore the orientation of the N-terminal aspartate residue in the enzyme activity site appears to be of crucial importance if aspartyl peptide hydrolysis is to occur. There do not however appear to be many general physical and chemical similarities between the different peptidases. These aspartyl-peptidases are further considered in the context of other peptidase literature in the later discussion section.

CHAPTER 5 GENETIC AND REGULATORY STUDIES OF *E. COLI* K12 ASPARTYL PEPTIDE HYDROLYSING ENZYMES

5.1 Introduction

Our understanding of the physiological roles of the *E. coli* K12 aspartyl-peptide hydrolysing enzymes would be enhanced by both genetic and regulatory studies of these peptidases. The absence of a mutant strain either overproducing or deficient in the *E. coli* K12 peptidase E activity precluded genetic studies on this peptidase. The availability however of mutant strains deficient in the aspartyl-peptide hydrolysing peptidase B, combined with a simple selection procedure for recombinants carrying this peptidase meant that this gene could be mapped on the *E. coli* K12 genome. Such mapping data would be essential for future cloning of the *pepB* gene which itself would facilitate regulatory and biochemical studies.

A preliminary estimate of the map position for the *pepB* gene was obtained from analysis of recombinant peptidase carrying strains isolated in conjugation experiments (Section 3.2.2.4). This estimated map position was localised further by interrupted mating (Miller, 1972) between an appropriate Hfr strain and a peptidase deficient recipient. Recombinants carrying the functional *pepB* gene were selected as colonies showing growth on the dipeptide Leu-Gly but not on Leu-Arg. The precise map position was determined using P1 transductional crosses. The frequencies of co-transduction between the *pepB* gene and nearby markers were calculated after transductional crosses and these frequencies converted to precise map distances (Wu, 1966).

The patterns of regulation of the synthesis of the E. coli K12 peptidase B and E genes were investigated by growing appropriate strains in both minimal and rich media and measuring the levels of each peptidase in cell extracts prepared at different phases of growth. Peptidase B and E activities were assayed with the growth substrates Leu-Gly and Asp-Leu respectively. Appropriate E. coli K12 strains were used to avoid possible difficulties arising from overlapping substrate specificities. Similar measurements of the levels of peptidase activities were made after growth of E. coli K12 strains on different carbon sources.

5.2 Results

5.2.1 Genetic Mapping of the Gene Encoding E. coli K12 peptidase B

5.2.1.1 Preliminary Localisation of the pepB Gene following analysis of Transconjugant Phenylalanine Auxotrophs

Studies carried out in Chapter 3 (section 3.2.2.4) involved in the construction of the peptidase deficient phenylalanine auxotrophs PN10-12, produced the observation that only 5 phenylalanine requiring recombinants were peptidase deficient after a 20 minute mating between the Hfr strain KA197 and the F^- peptidase deficient strain PN4. All other phenylalanine auxotrophs gained the ability to utilise the dipeptide Leu-Gly as a source of these strains' leucine requirement, indicating the transfer of a Leu-Gly hydrolysing peptidase during mating.

The leucyl-peptide utilisation properties of 3 of these Leu-Gly utilising strains designated , PN13-15, were further investigated and these were all found to utilise Leu-Gly but not Leu-Arg as a source of required leucine, a growth phenotype consistent with the presence of peptidase B (Chapter 3 Table 5). Analysis of the peptidase profiles of cell extracts from these peptidase carrying recombinants, using the PAGE gel assay system, revealed a band of Leu-Gly hydrolysing activity mobility 0.3, corresponding to peptidase B , confirming that this peptidase had been transferred to recipient strains during mating.

The point of origin of transfer for genes from the Hfr strain KA197 lies at around 63 minutes on the E.coli K12 genome (Brooks-Low 1987) and genes are transferred in an anti-clockwise direction from the origin of transfer.
The data from the KA197/PN4 mating experiment indicates that the peptidase B encoding gene lies close to the PheA marker at 56.8 minutes but the presence of a small number of phenylalanine auxotrophs deficient in peptidase B suggests that this pepB gene lies distal to the Phe A marker away from the point of origin.

5.2.2. Mapping of the pepB gene by Interrupted Mating

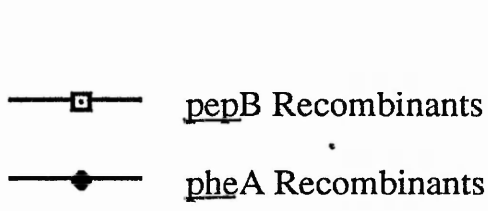
The method of interrupted mating was used to further localise the map position of the pepB marker gene. The E.coli K12 Hfr strain KL16, prototrophic for phenylalanine and presumed wild-type for the known

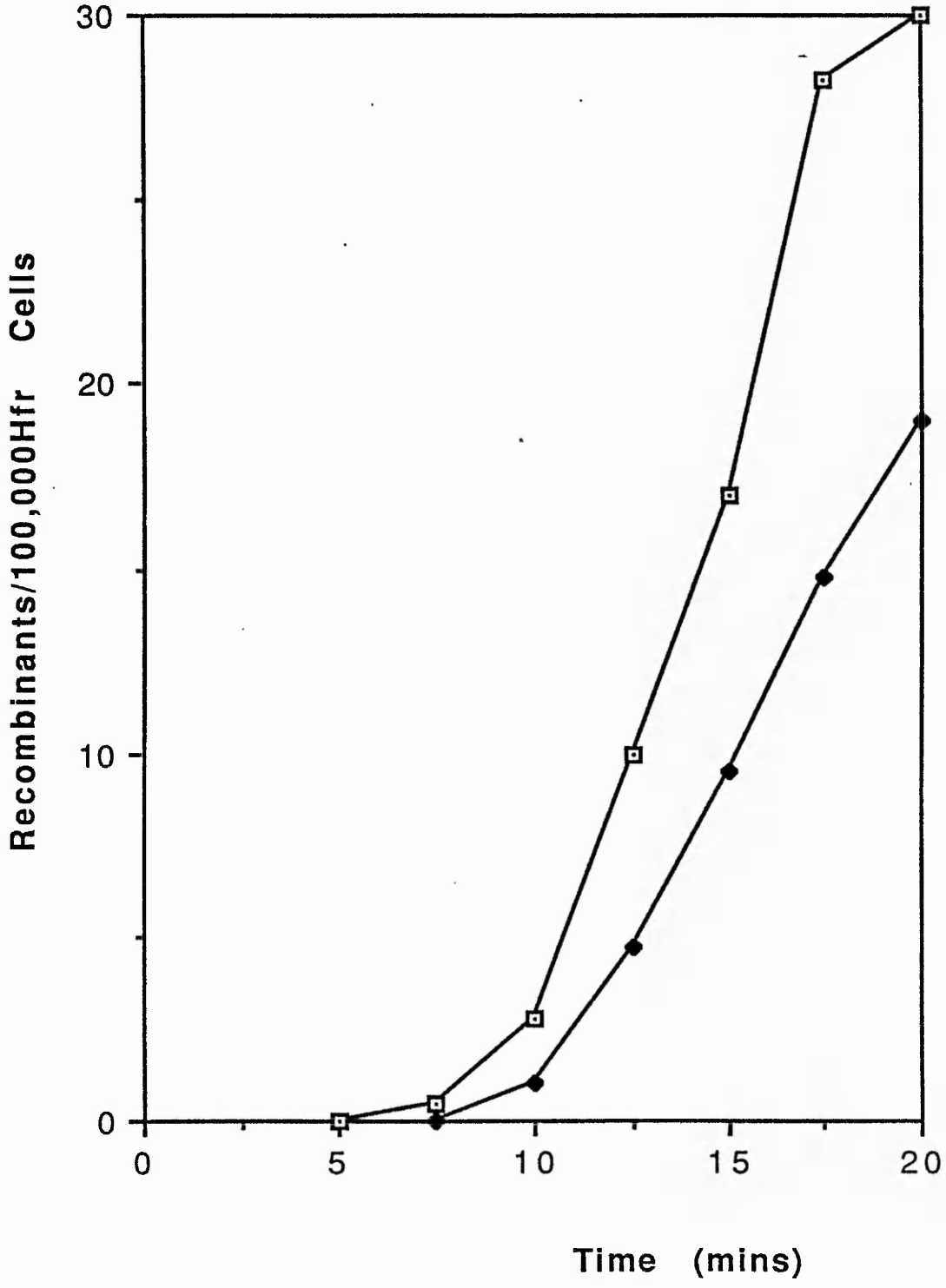
peptidase enzymes, and sharing the same origin of transfer as strain KA197 (at around 63 minutes), was mated with the peptidase deficient phenylalanine auxotroph PN31. Mating mixture was sampled every 2.5 minutes and after vortexing, peptidase carrying and phenylalanine prototrophic recombinants were selected on appropriate selective plates. Frequencies of occurrence of each type of recombinant were plotted against mating times (Fig. 25). Analysis of the resulting graph showed that transfer of the pepB gene preceded transfer of the PheA gene and at all sampling points pepB$^+$ recombinants occurred at higher frequencies than PheA$^+$ recombinants. The pheA and pepB genes also appeared to be closely linked on the genome as shown by the small differences in the start times for gene transfer (approximately 7.5 minutes and 10 minutes respectively). The data suggests that pepB lies within 2-3 minutes of the PheA gene on the E. coli K12 genome, and 5-6 minutes from the KL16 origin of transfer, in between this origin of transfer and the PheA gene.

5.2.1.3 Fine Mapping of the PepB Gene using P1 Transductional Crosses

The data from mating experiments indicated that the gene encoding the E. coli K12 peptidase B was located close to the PheA marker. Experiments were therefore carried out to determine the degree of linkage and map distance between these markers. P1 transductional crosses were carried out

Figure 25. Determination Of PheA And PepB Encoding Recombinants After Interrupted Mating




30
20
10
0
0
5
10
15
20
Recombinants/100,000Hfr Cells
Time (mins)

using the pheA$^+$pepB$^+$ strain CM17 as donor culture and the pheA$^-$pepB$^-$ strain PN31 as recipient. PheA$^+$ colonies were selected and 55 out of 200 PheA$^+$ colonies tested were shown to have acquired the pepB$^+$ growth phenotype, representing a co-transduction frequency of 27.5% corresponding to a genetic map distance of 0.72 minutes between these markers.

To further localise the pepB gene, 3 factor crosses were carried out using PheA$^+$pepB$^+$ donor strains carrying genetic markers mapping near PheA on the E. coli K12 genome (Bachmann, 1983), GlyA$^-$ (strain AT2457 requiring glycine), RecA$^-$::Tn10 (SA317; UV sensitive, tetracycline resistant), PdxJ (AT 3208 requiring pyridoxine), and PUVA^r (JH101; resistant to Psoralen-near UV light treatment), and using strain PN31 as recipient. PheA$^+$ recombinants were selected and linkage frequencies with pepB and other unselected markers determined (Table 18). Strain SA317 was also used as a donor strain in a 3-factor cross with PN31 as recipient. Initial selection in these crosses was for tetracycline resistance.

Summing the Table 1 data for all P1 transductional crosses involving initial selection for pheA$^+$ recombinants, 389 out of 1,400 PheA$^+$ recombinants tested carried the pepB$^+$ gene giving a linkage frequency of 27.7% and a calculated map distance between the 2 markers of 0.7 minutes. The linkage frequency between RecA :: Tn10 and pepB was

Table 18 Co-transduction frequencies from 3-factor P1 (*vir*) Transductional Crosses

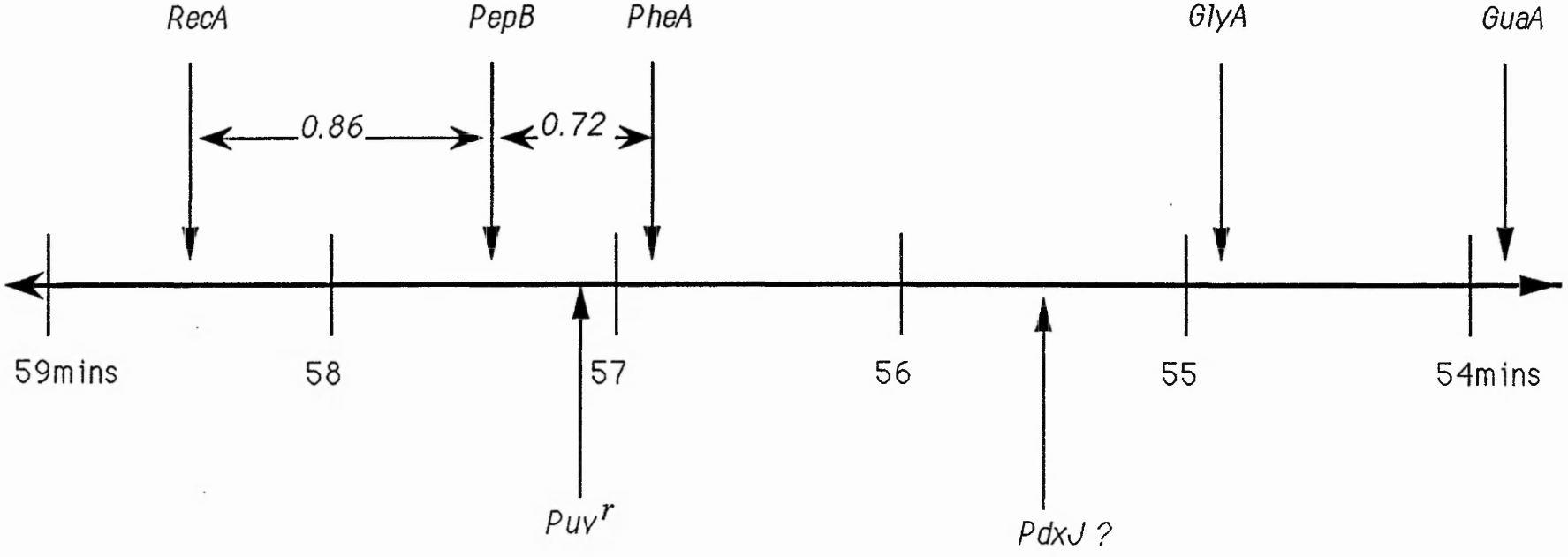
Donor	Recipient	Selected Class	No of Colonies Tested	Unselected Class	No	Frequency (%)
AT 2457	PN31	*PheA*$^+$	400	pepB$^+$Gly$^+$	98	24.5
(*GlyA*)				pepB$^+$Gly$^-$	0	0.0
				pepB$^-$Gly$^+$	301	75
				pepB$^-$Gly$^-$	1	0.25
SA317	PN31	*PheA*$^+$	400	pepB$^+$Tet^r	5	1.3
(*RecA::Tn10*)				pepB$^+$Tet^s	116	29.0
				pepB$^-$Tet^r	0	0.0
				pepB$^-$Tet^s	279	69.8
JH101	PN31	*PheA*$^+$	30	pepB$^+$PUVA^r	13	43.3
(*PUVA*^{r})				pepB$^+$PUVA^s	0	0.0
				pepB$^-$PUVA^r	3	10.0
				pepB$^-$PUVA^s	14	46.7
AT3208	PN31	*PheA*$^+$	400	pepB$^+$PdxJ$^+$	112	28.0
(*PdxJ*)				pepB$^+$PdxJ$^-$	0	0.0
				pepB$^-$PdxJ$^+$	264	66.0
				pepB$^-$PdxJ$^-$	24	6.0
SA317	PN31	*Tet*^{r}	400	pepB$^+$PheA$^+$	3	0.75
(*RecA Tn10*)				pepB$^+$PheA$^-$	72	18.0
				pepB$^-$PheA$^+$	0	0.0
				pepB$^-$PheA$^-$	325	81.25

determined at 18.75% from 400 colonies tested, indicating a map distance of 0.86 minutes between these markers. The pepB gene was also co-transduced with the PUVA^r gene but no co-transduction was observed between pepB and either the GlyA or PdxJ genes.

From the tabulated data for SA317 3-factor crosses a co-transduction frequency of 1% was determined between PheA and RecA::Tn10 genes indicating a map distance of 1.57 minutes between these genes. The linkage between PheA and the PdxJ genes was determined at 6.0% equivalent to a map distance of 1.2 minutes between these 2 genes. The map distance between pheA and the PUVA^r gene was similarly determined at 0.38 minutes, a figure subject to some error as only 30 Phe$^+$ colonies were tested for PUVA^r in the appropriate cross. The Gly$^-$ marker was calculated to lie at 1.66 minutes from the PheA gene.

The genes encoding PheA, GlyA, PdxJ, RecA have all been mapped on the E. coli K12 genome at positions 56.7, 54.8, 55.6 and 58.3 minutes respectively (Bachmann, 1983) and the position of the PUVA^r gene has been mapped at 57.2 minutes (J Holland pers comm.). Combining these map positions with the relative map distances calculated from co-transduction experiment places pepB at approximately 57.5 minutes on the E. coli K12 genome (Fig. 26), lying between RecA and PheA. All pepB$^+$ transductants from the JH101 PUVA^r cross

Figure 26. Part Of The E.coli K12 Genetic Map Showing The Position Of The PepB Gene And Nearby Markers



are *PUVA*^{r} confirming the calculated position of *pep* B as proximal to *Rec*A with respect to the *PUVA*^{r} gene.

Final confirmatory transductional crosses were carried out using the *pepB*$^+$*PheA*$^+$ strain SA317 as donor culture and strain PN31 as recipient culture with initial selection for *pepB*$^+$ recombinants. 117 out of 400 *pepB*$^+$ recombinants were found to be *PheA*$^+$ giving a linkage frequency of 29.25% and a calculated map distance of 0.68 minutes between these markers.

5.2.2 Studies on the Regulation of the synthesis of the *E. coli* K12 Aspartyl-Peptide Hydrolysing Enzymes, Peptidases B and E

5.2.2.1 Analysis of the Effects of Different Growth Media on Levels of Peptidase B and E activities

E. coli K12 strains PN31 (*pepE*$^+$) and PN56 (*pepE*$^+$*pepB*$^+$) were used in regulatory studies in order to allow measurement of both peptidase B and E activities and to remove difficulties presented by the overlapping substrate specificities of these peptidases. Strains were grown up overnight in appropriate growth medium then inoculated into a fresh batch of the same medium (Table 19). After incubation cells were harvested in both exponential phase ($O.D._{600}$ = 0.6; = 4 x 10^8 cells/ml) and stationary phase ($O.D._{600}$ = 1.0; = 2 x 10^9 cells/ml). Specific enzyme activities were determined at each growth phase using the

Table 19 The effects of Rich and Minimal Media on Levels of Peptidase B and E in *E. coli* K12

Medium	Exponential Phase (0.6) Enzyme Activity[a] peptidase B	peptidase E	Stationary Phase (1.0) peptidase B	peptidase E
M9 + amino acids	14.2	12.0	18.9	20.1
M9 + Leu-Gly	14.1	-	16.2	-
M9 + Asp-Leu	15.0	12.2	19.4	15.3
Nutrient Broth	15.0	12.1	22.1	20.4

[a] Enzyme activities are given in units mg protein^{-1} x 1,000 for each peptidase with its respective assay substrate.

HPLC quantitative assay system for measurement of enzyme activity, in the case of peptidase E by assaying Asp-Leu hydrolytic activity in PN31 crude extracts and in the case of peptidase B by assaying Leu-Gly hydrolytic activity in PN56 crude extracts (Table 19).

Peptidase B and E activities remained largely constant in the different media at both exponential and stationary phase. A fractionally higher level (5-14% increase) of peptidase B activity was observed in both nutrient broth and Asp-Leu supplemented M9, when compared with amino acid supplemented M9 at each growth phase.

Stationary phase cells were found to contain higher levels of both peptidase B and E activities in all media used and though the observed levels of increase were not substantial (from 14-70% increases over exponential phase levels) they were observed consistently and this difference in activity levels was considered therefore to be of possible physiological significance.

5.2.2.2 Analysis of the Effects of Carbon Source on Levels of Peptidase B and E activities

E. coli K12 strains PN31 and PN56 were grown up overnight in minimal medium supplemented with 0.2% glycerol or 0.2% glucose as carbon source. Cells were harvested by centrifugation and inoculated into fresh medium containing

the same carbon source. After incubation, cells were harvested at pre-determined optical densities representing exponential and stationary phases of growth on each carbon source. Sonicated crude cell extracts were prepared and levels of protein, and levels of peptidase B and E activity determined (Table 20). Peptidase B activity was found to be substantially higher at both exponential and stationary phase when cultures were grown in glycerol containing media rather than glucose media. Peptidase E activity remained higher than the glucose equivalent at exponential phase but was higher in glucose medium at stationary phase. Activities of both peptidases were observed to be greater in stationary phase cultures than in exponential cultures irrespective of the growth medium. The reduction of peptidase B activity observed in ~~glycerol~~ glucose grown cultures when compared with ~~glucose~~ glycerol grown cultures could possibly be related to the acidification of culture media mediated by glucose degradation products. The final pH values for glucose grown cultures (at $O.D._{600}$ 1.0), were 7.2 for both strains PN31 and PN56, whereas for glycerol grown cultures the final pH ($O.D._{600}$ 0.75) remained at pH 7.5. Such a relationship could not be confirmed as other factors derived from the pleiotropic influence of cellular glucose metabolism could be effecting levels of peptidase B activity. The observed acidification of the growth medium does not appear to significantly effect levels of peptidase E activity.

Table 20 The Effects of Different Carbon Sources on Levels of Peptidase B and E activity

Carbon source	Exponential Phase (0.6) Enzyme Activity[a] peptidase B	peptidase E	Stationary Phase (1.0) peptidase B	peptidase E
Glycerol	29.4	14.0	31.2	16.2
Glucose	14.1	12.0	19.4	21.2

[a]Exponential Phase cultures harvested at O.D. 0.6 in glucose grown cultures and at O.D. 0.5 in glycerol grown cultures.

[b]Stationary Phase cultures harvested at O.D. 1.0 and O.D. 0.75 in glucose grown and glycerol grown cultures respectively.

[c]Enzyme activities are given in units mg-protein^{-1} x 1,000 for each peptidase with its respective assay substrate.

5.3 Discussion

Using both mating experiments and P1 transductional studies, the gene encoding *E. coli* K12 peptidase B has been mapped at position 57.4-57.5 minutes on the *E. coli* K12 genome. The region of the K12 genome from 57-58 minutes is poorly characterised and this fact reduced the number of possible marker genes available for fine mapping in P1 linkage experiments with the *pepB* gene. The availability of more selectable markers in this region would have allowed greater accuracy and confidence in the precision of map determinations. The fact however that more than 1,000 *PheA*$^{+}$ transductant colonies were tested for the presence or absence of the *pepB* gene provides grounds for confidence both in the calculated linkage frequencies and the resulting designated map position. The absence of any observed co-transduction between *pepB* and *PdxJ/GlyA* genes combined with the observation of co-transduction between *pepB* and both the (*PUVA*)^{r} encoding gene and *RecA*::*Tn10* marker confirmed the location of *pepB* between the *PheA* gene at 56.8 mins and the Hfr KL16 origin of transfer at 63 minutes.

Regulatory studies demonstrated no significant induction of *E. coli* K12 peptidase B or peptidase E activities on growth of *E. coli* K12 strains in rich peptide containing medium, indicating that both peptidases are synthesized constitutively. The observation of significantly increased levels of both peptidase activities

in stationary phase cells when compared with exponential cells, may reflect enhanced levels of protein turnover previously reported in stationary phase cells (Mandelstam, 1960).

CHAPTER 6 EXPERIMENTS AIMED AT SYNTHESISING THE DIPEPTIDE SWEETENER ASPARTAME

6.1 Introduction

The initial aim of this research project was to identify and establish a novel route for the synthesis of the valuable dipeptide sweetener aspartame. No such route was identified.

The current short chapter summarises briefly the approaches adopted and the conditions used in efforts to synthesise the sweetener and considers why no synthesis was observed in the experiments carried out.

The main thrust of the synthesis effort was devoted to reversing the process of peptidase catalysed hydrolysis of aspartame. The observation in the literature of a number of different peptide syntheses involving reverse hydrolysis catalysed by protease enzymes (for example Kullmann, 1980), in particular the thermolysin mediated synthesis of aspartame from derivatised amino acid precursors (Isowa et al., 1979) suggested that such an effort might prove fruitful. The use of N-terminal L-α-aspartyl peptide hydrolysing enzymes would, it was considered, facilitate peptide synthesis as such peptidases would have the potential to bind the aspartame precursors aspartate and phenylalanine methyl ester in the correct sequence for sweetener synthesis.

The first experiments carried out used whole bacterial cells as catalysts for sweetener synthesis. Whole bacterial cells had been reported to catalyse the synthesis of aspartame from amino

acid precursors (Ajinomoto Corp. 1985) and in the first instance it was aimed to repeat these experiments using our own bacterial strains and to develop this synthesis process. It did not however prove possible to repeat the reported syntheses.

Crude cell extracts were prepared from bacterial strains previously demonstrated as carrying aspartyl-peptide hydrolysing enzymes. Levels of aspartyl-peptide hydrolysing activity were determined in each cell extract and samples of each extract incubated with dipeptide precursors under varying conditions. Partially purified peptidases obtained in the work described in Chapter 4 were also incubated under appropriate conditions with dipeptide precursors. Factors such as enzyme concentration, substrate concentrations, hydrogen ion concentration and solvent type were varied in order to promote dipeptide synthesis. Performing the synthesis reaction in the presence of the water miscible solvents glycerol or 1,4,-butanediol would theoretically shift the equilibrium position from peptide breakdown to synthesis by reducing the concentration of water available for hydrolysis. The use of a biphasic ethyl acetate: water solvent system would promote synthesis if product aspartame was more soluble in the ethyl acetate phase than the aqueous phase where synthesis would occur. Under the different conditions used and with both crude cell extracts and purified peptidases, no dipeptide synthesis was observed as shown by analysis of product mixtures by either TLC or HPLC methods.

6.2 Results

6.2.1 Experiments aimed at the Synthesis of the Dipeptide Sweetener Aspartame

6.2.1.1 Experiment using whole cells

A single patent (Ajinomoto Corp. 1987) described the synthesis of aspartame using whole bacterial cells incubated under appropriate conditions with the aspartame precursors L-aspartate and L-phenylalanine methyl ester. Bacterial strains were grown up in 'aspartame synthesis medium' (see Materials and Methods) and attempts made to repeat the aspartame synthesis using identical reaction conditions but with bacterial strains from the Trent Polytechnic culture collection in place of those strains described in the patent. These efforts proved unsuccessful and no aspartame product could be detected by thin layer chromatographic analysis. Varying the cell concentration had no effect on synthesis (Table 21).

TLC chromatographs (not shown) of product mixtures, separated in the previously described solvent (1), (see Table 2), showed the presence of an amino acid residue in addition to the aspartame precursors, of mobility corresponding to phenylalanine, indicating probable breakdown of phenylalanine methyl ester to phenylalanine. Such breakdown of phenylalanine methyl ester could effect the desired synthesis of aspartame. Further synthesis reactions were therefore set up using whole bacterial cells

Table 21 Bacterial strains and experimental conditions employed for the attempted synthesis of Aspartame using whole bacterial cells

Bacterial strain	Weight of cells in Incubation Mixture(g)	[Phe]/ [PheOMe] (mM)	[Asp] (mM)	Incubation Time (hrs)	Incubation Temperature (°)
E. coli CM17	0.2	4	2	16	30
	1.0	"	"	24	"
	2.0	"	"	24	"
A. faecalis	0.2	"	"	16	"
	1.0	"	"	24	"
	2.0	"	"	24	"
F. denitrificans	0.2	"	"	16	"
	1.0	"	"	24	"
	2.0	"	"	24	"
C. fascians	0.2	"	"	16	"
	1.0	"	"	24	"
	2.0	"	"	24	"
F. lucecoloratum	0.2	"	"	16	"
	1.0	"	"	24	"
	2.0	"	"	24	"
B. subtilis	1.0	"	"	24	"
	2.0	"	"	24	"

and aspartate and phenylalanine as substrates in an effort to synthesise the dipeptide Asp-Phe (Table 21). No Asp-Phe product was detected after TLC analysis of product mixtures.

6.2.1.2 Experiments using Crude Cell Extracts

In the absence of aspartame synthesis catalysed by whole cells it was decided to utilise crude cell extracts containing N-terminal L-α-aspartyl peptide hydrolysing enzymes, in order to catalyse synthesis of sweetener, potentially by reverse hydrolysis. Sonicated crude cell extracts were prepared from E. coli CM17, A. calcoaceticus and B. subtilis cultures grown up in nutrient broth. Levels of aspartyl peptidase were estimated in each cell extract using the TLC based assay with Asp-Phe as assay substrate, and samples of extract incubated with both aspartame and Asp-Phe precursors under a variety of conditions (Table 22); pH, substrate concentrations and solvent systems were varied but under no conditions was synthesis of dipeptide observed as determined by TLC and HPLC analysis. In some experiments crude cell extracts were concentrated 10-fold to enhance enzyme activity but the presence of these raised levels of enzyme activity in incubation mixtures and had no effect on dipeptide synthesis. TLC analysis again revealed the presence of phenylalanine in all Asp/PheOMe product mixture indicating the possible presence in crude cell extracts of amino acid esterase activities.

Table 22 Conditions employed for the attempted synthesis of Aspartame using crude bacterial cell extracts

Organism	Reaction Volume (mls)	Units of Activity[a]	[Phe[/]PheOMe] (mM)	[Asp] (mM)	Solvent	pH	Incubation Temperature
E. coli CM17/	10	0.5	10	5	dH_2O	5.5/7.0/9.0	30°C
A. calcoaceticus/	"	"	4	2	"	"	"
B.subtilis	"	0.05	10	5	"	"	"
	"	"	4	2	"	"	"
	"	0.5	1.10	5	75% Ethyl Acetate	7.0	"
	"	"	"	"	85% 1,4-Butanediol	"	"
	"	"	"	"	60%,80%,90% Glycerol	5.5/7.0/9.0	"

[a]1 unit of activity is defined as the amount of enzyme required to hydrolyse 1 mol of Asp-Phe per minute under standard conditions.

6.2.1.3 Experiments using Partially Purified Peptidase Preparations

Partially purified bacterial peptidases were also used as catalysts for aspartame synthesis under conditions similar to those used with crude cell extracts (Table 23). Neither aspartame or Asp-Phe synthesis was detected in these experiments as shown by TLC analysis. No phenylalanine was generated from PheOMe contained in the aspartame synthesis reaction mixture indicating that aspartyl-peptidases would not hydrolyse PheOMe.

6.3 Discussion

The occurrence of aspartame synthesis on mixing bacterial whole cells and amino acid precursors is an unexpected phenomenon and no report similar to that from Ajinomoto on the synthesis of aspartame or other peptides using whole bacterial cells have been found in the literature. The mechanism of this synthesis was not discussed in the Ajinomoto patent and it is difficult to envisage the mechanism for such a synthesis considering the observed presence in bacterial strains of aspartame degrading peptidases. The fact that the synthesis of aspartame could not be repeated in the work carried out in this project may possibly relate to the use of our own culture collection strains in synthesis experiments rather than Ajinomoto strains.

The experiments reported in this chapter using both crude cell extracts and purified peptidase enzymes as catalysts for

Table 23 Conditions used for attempted synthesis of Aspartame using Partially Purified Peptidases

Partially Purified Enzyme	Reaction Volume (ml)	Units of Activity[a]	[PheOMe] (mM)	[Asp] (mM)	Solvent	pH	Incubation Temperature
E. coli K12 Peptidase B/	1	0.02	10	5	dH_2O	5.5/7.0/9.0	30°C
E. coli K12 Peptidase E/	4		2	"	"	"	
A. calcoaceticus ANA Hydrolysing Peptidase			10	5	"	"	"
			4	2	"	"	"
			10	5	75% Ethyl Acetate	"	"
			"	"	85% 1,4-Butanediol	"	"
			"	"	60%/80%/90% Glycerol	"	"

[a] 1 unit of activity is defined as the amount of enzyme required to hydrolyse 1 µmol of the appropriate enzyme substrate under standard conditions.

aspartame synthesis from amino acid precursors, generated no sweetener product, and indicate that peptidase catalysed reverse hydrolysis is not a promising route for dipeptide syntheses. The equilibrium position appears to favour peptide hydrolysis to such a degree that under the conditions used no synthesis can be observed. Even reducing the amount of water available for hydrolysis will not shift equilibrium far enough towards peptide synthesis to allow product formation, while raising non-water solvent concentration to 100% in order to completely prevent hydrolysis is likely to completely denature peptidase enzymes (Martinek & Semenov, 1981).

Protease catalysed synthesis of peptidases described in the literature used derivatised amino acid substrates for peptide synthesis and the derivatising groups appear to activate the amino acid residues involved in peptide bond formation reactions (Kullman, 1980). Thus it may be necessary to derivatise amino acid residues before peptidase catalysed synthesis of aspartame can be demonstrated. The fact however that aspartame cannot be synthesised directly from precursor amino acid residues does not rule out the possibility that tripeptidases or oligopeptidases might be used for peptide synthesis as the energy required for synthesis of tripeptides from dipeptide and amino acid substrates is much lower than that required for dipeptide synthesis from amino acids (Borsook, 1953).

CHAPTER 7 DISCUSSION

7.1 A comparative look at the Aspartyl Peptide Hydrolysing Enzymes

Studies over the past 20 years have shown that *E. coli* K12 produces at least 10 different peptidases with varying specificities and other properties (Miller, 1987). In the work described in this thesis, 2 of the previously identified peptidases, peptidases B and Q, have been identified as hydrolysing N-terminal L-α-aspartyl peptides, the former hydrolysing a broad range of aspartyl-peptide substrates and the latter hydrolysing only Asp-Pro. A further enzyme designated peptidase E, previously unreported in this organism, was also identified as hydrolysing a range of N-terminal L-α-aspartyl-peptides. The *E. coli* K12 peptidases N, A and D, previously implicated in the hydrolysis of these peptides were shown not to be involved in this process. This section looks at the range of peptidases involved in the hydrolysis of L-α-aspartyl peptides and compares the data obtained for the aspartyl peptide hydrolysing enzymes, peptidases B and E, partially purified in this thesis, with the properties ascribed to the other microbial peptidases identified in *E. coli* K12 or the closely related *S. typhimurium* LT2. This is done with a view to considering the similarities and differences in regulation, catalytic mechanism, physical structure and functions of these enzymes. The ANA hydrolysing peptidase identified in *A. calcoaceticus* is also compared with the other microbial peptidases.

The 3 aspartyl-peptide hydrolysing activities, B, E and Q

were ascribed this specificity by a combination of growth tests and biochemical assays. The data presented also indicated the possible presence in E. coli K12 of additional aspartyl-peptide hydrolysing activities as evidenced by the lack of a mutant defective in the ability to use aspartyl-peptides as sole sources of required amino acids and the lack of mutants identified as deficient in aspartyl-peptidase activity as determined by a colorimetric assay. The lack of such mutants could however also be due to the non-viability of aspartyl-peptidase deficient mutants. If additional aspartyl-peptidase activities were present in E. coli K12 then activities could not, unlike other peptidase activities, be demonstrated after activity staining of crude cell extracts separated by polyacrylamide gel electrophoresis, nor were these activities observed during purification of peptidases B and E. This could possibly be due to the sensitivity of these peptidases to the procedures used for generating cell extracts. The presence of additional aspartyl-peptide hydrolysing enzymes supplementing the activities of peptidases B, E and Q would mirror to a degree the system of aspartyl-peptide hydrolysing enzymes as described in the closely related S. typhimurium LT2. In this bacterial strain 2 aspartyl-peptide hydrolysing enzymes have been identified in addition to peptidases B and E (Carter & Miller, 1984). However as peptidase Q was not identified as degrading aspartyl peptides in this organism, and no such additional aspartyl-peptide activities were identified in E. coli K12, the evidence tends to indicate that slightly different aspartyl-peptide degrading systems pertain in the 2 organisms.

Regulatory studies showed that both peptidases B and E are synthesised constitutively in E. coli K12. This data runs parallel with other studies on the regulation of peptidase synthesis in this organism. Peptidase N has been shown to be synthesised constitutively (McAman et al., 1982; Ladzunski et al., 1975b) as has the equivalent S. typhimurium LT2 peptidase T (Strauch et al., 1983), providing preliminary evidence that peptidase synthesis may be regulated by similar mechanisms.

The levels of both peptidase B and E activities were found to be higher in stationary phase cells than in exponential cells. The physiological significance of this increase in peptidase activity is not clear. A number of workers have demonstrated that protein turnover occurs at higher rates in non-growing E. coli cells than in exponential phase, growing cells (Rotman & Spiegelman, 1954; Hogness et al., 1955; Mandelstam, 1958). An increase in cellular peptidase activity may be necessary to accommodate these higher levels of protein turnover. Contradictory findings have come from investigations of the levels of peptidase N activity during the cell cycle. In one study it was reported that the specific activity of peptidase N remained constant (McAman et al., 1982) whereas a further study claimed higher levels of peptidase N activity in stationary phase cells (Yang & Somerville, 1976). Levels of S. typhimurium LT2 peptidase T have been reported to be higher in late exponential and early stationary phase cells while no such difference was observed with the Salmonella dipeptidyl carboxypeptidase (Strauch

et al., 1985). Variation in sets of experimental results may possibly relate to the slightly different growth media and conditions used by different groups of workers.

Further contradictory data on the levels of peptidase activities during the E. coli K12 growth cycle has been produced from studies of the relationship between different carbon sources and peptidase activity ~~level~~s. In an early study it was shown that in a growth medium containing peptone as the sole source of carbon, levels of E. coli K12 dipeptidase activity were the same as those in peptone medium supplemented with 0.6% glycerol (Simmonds, 1970). Replacement of the glycerol supplement with 0.6% glucose resulted in higher levels of Leu-Gly and Phe-Gly activity in exponential phase which decreased rapidly at the onset of stationary phase. The data presented in this thesis demonstrates some small variation in peptidase B and E activity levels when E. coli K12 strains were grown with the different carbon sources, glycerol and glucose, yet the extent of these effects was dependent on the growth phase sampled and was never significant. Levels of E. coli K12 peptidase N have been reported to be unchanged on varying carbon source (McAman et al., 1982). However other studies report significant variations in peptidase N activity levels dependent on carbon source (Murgier & Gharbi, 1982; Gharbi et al., 1985). Levels of S. typhimurium LT2 peptidase T activity were reported to be unaffected by varying carbon sources (Strauch et al., 1984).

The effect of carbon starvation on levels of peptidase B and E activities in E. coli K12 cells was not investigated in this thesis. Carbon starvation is reported to increase levels of protein turnover in E. coli K12 (Mandelstam, 1960) and so it might be expected that the levels of cellular peptidase activity would increase, as these enzymes are involved in the terminal steps of protein degradation (Yen et al., 1980a). 2 separate studies however have reported no increase in the levels of peptidase N activity when exponential phase cells are starved for carbon (McAman et al., 1982; Ladzunski et al., 1975) and no increase in peptidase T activity levels was observed when S. typhimurium LT2 cells were starved for carbon (Strauch et al., 1985).

Further studies on the regulation of the E. coli K12 aspartyl-peptidase hydrolysing enzymes B and E will be required if a complete comparison with the regulation of other peptidases is to be made. This would require studies of the effects of phosphate limitation, nitrogen starvation, nutrient limitation and anaerobiosis on levels of peptidase B and E activities and would also require resolution of some of the apparent contradictions in the published regulatory literature for other peptidases. For example the levels of peptidase N have been shown in one study to be unaffected by phosphate limitation (McAman et al., 1982) however several other studies report an increase in peptidase N activity under these conditions (Ladzunski et al., 1975b; Gharbi et al., 1985; Foglino &

Ladzunski 1987). All reports in the literature state that nitrogen starvation has no effect on levels of peptidase N (McAman et al., 1982; Ladzunski et al., 1975b).

Studies on nutrient limitation effects on the level of peptidase T activity in S. typhimurium LT2 using strains with pepT MudI (lac,Ap) insertions encoding β-galactosidase from a pepT promoter, showed no changes in the levels of β-galactosidase activity with limiting glucose, ammonia or leucine nor were elevated levels of β-galactosidase expressed from the pepT promoter before or after cessation of growth (Strauch et al., 1985). These workers also tested the effect of anaerobiosis on expression of β-galactosidase from the pepT lac fusion, and found levels of peptidase activity comparable to those of stationary phase cells from aerobic cultures. They concluded that oxygen limitation during late-exponential phase caused increased pepT expression in aerobic cultures. This assertion was confirmed by direct measurement of peptidase T activity levels under the same conditions. The demonstration of peptidase T regulation by oxygen levels in S. typhimurium led this same group to isolate mutations in 2 loci, designated oxrA and oxrB (oxygen regulation) which prevented induction of the pepT locus. The oxrA locus was found to be homologous to the fnrA locus of E. coli involved in anaerobic regulation and the process of cell division. The expression of E. coli K12 pepN has also been shown to be stimulated by conditions of anaerobiosis (Gharbi et al., 1985; Foglino & Ladzunski, 1987) and may be regulated in a

similar manner to pepT. Also of interest is the recent report that S. typhimurium LT2 peptidase T expression can be stimulated by osmotic stress (de Rienhdt, 1988).

Although the regulatory data for E. coli K12 and S. typhimurium LT2 peptidases is far from complete it seems reasonable to suggest that most of these peptidases, including the aspartyl-peptide hydrolysing enzymes B and E, are constitutively synthesised, but their production is regulated by global regulatory networks, in particular those co-ordinating the cellular response to anaerobiosis and phosphate limitation. This scenario however remains far from certain. It is possible for example that particular peptidases such as the S. typhimurium peptidase T play a special role in the response to anaerobiosis by for example processing a signal peptide molecule and hence are regulated in a manner contrasting with that of other peptidases. Similar roles and patterns of regulation may yet to be ascribed to other peptidases for which such detailed regulatory studies such as those with peptidase T from S. typhimurium LT2 have yet to be carried out.

The E. coli K12 aspartyl-peptidase hydrolysing enzymes were shown in chapter 4 to have markedly different molecular mass values. The E. coli K12 peptidase B has an M_r of around 230kd, a value corresponding to that previously reported for this enzyme (Hermsdorf & Simmonds 1980) whereas peptidase E has an M_r of 35kd. This mass variation is seen throughout the E. coli K12

peptidases, with no discernible common structural features as yet apparent. Peptidase A is a hexameric molecular of M_r 323kd (Vogt, 1970), the dipeptidase peptidase D is a dimer M_r 108kd (Klein et al., 1986) whereas the well characterised amino acid napthylamide hydrolysing peptidase N appears to be monomeric of M_r 100kd (Yang & Somerville, 1976; Foglino et al., 1986).

It is of interest to note the wide variation in mobilities of the different peptidases when separated on 7.5% polyacrylamide gels. The 35kd E. coli K12 peptidase E gives a mobility of 0.5 (this thesis) while the 'equivalent' S. typhimurium LT2 enzyme, reportedly of similar M_r has a mobility of 0.7 on 7.5% gels (Carter & Miller, 1984). This variation points to a substantial difference between these 2 enzymes in either structure or overall charge. The 108kd E. coli K12 peptidase D gives a mobility of 0.48 (Miller & Schwartz, 1978; this thesis) whereas the 158kd S. typhimurium LT2 peptidase D is reported to have mobility 0.52 (Miller & Mackinnon, 1974) and the 100kd K12 peptidase N has mobility 0.6. In addition the dimeric S. typhimurium LT2 peptidase T, M_r 80kd, has a mobility of 0.4 (Strauch et al., 1985).

The observed variation in peptidase molecular mass probably relates to the varying substrate specificities of these peptidases though whether a particular structure is an absolute requirement for a particular range of peptidase specificity can only be a matter for speculation at this stage. The large broad

specificity and aspartyl-peptide hydrolysing, peptidase B, hydrolyses both dipeptides and tri-peptides with substrates ranging from the hydrophobic dipeptide Leu-Leu and the tripeptide Leu-Gly-Gly (Miller & Schwartz, 1978) to the acidic dipeptide Asp-Glu. Separate active sites may be required to accommodate the catalysed hydrolysis of such diverse substrates. Evidence for distinct binding sites has been obtained for E. coli K12 peptidase N (Yang & Somerville, 1976). Competitive studies using the different peptide substrates will be required to determine whether the different peptide substrates compete for the same sites on the peptidase B enzyme molecule.

The E. coli K12 peptidase E hydrolyses only N-terminal L-α -aspartyl peptides. Neither glutamyl dipeptides or C-terminal aspartyl dipeptides would serve as substrates for the enzyme. In this respect the E. coli K12 peptidase E is identical to the S. typhimurium LT2 peptidase E (Carter & Miller, 1984). Relative specificities towards particular aspartyl-peptides varied slightly between these 2 enzymes. Comparison of substrate affinities shows that E. coli K12 peptidase E has lower affinity for the dipeptide Asp-Phe although other specificities were broadly similar. The major difference between the 2 enzymes lies in the ability of S. typhimurium LT2 peptidase E to hydrolyse the dipeptide Asp-Pro. Contrary to the S. typhimurium LT2 data, E. coli K12 strains carrying peptidase E were shown in this thesis to be unable to utilise Asp-pro as a source of aspartate whereas strains carrying peptidase Q gained this characteristic,

indicating that peptidase Q hydrolyses Asp-pro whereas E. coli K12 peptidase E does not.

Peptidase E has been shown in this thesis to be the only E. coli K12 peptidase, the activity of which appears not to be inhibited by the presence of metal ions or metal ion chelating agents, as demonstrated through both qualitative gel assays and quantitative studies. In this respect the E. coli K12 enzyme is similar to that identified in S. typhimurium LT2. One possibility is that these peptidase E enzymes catalyse hydrolysis of their substrates by a completely different mechanism from those employed by other peptidases. However addition of serine protease or sulphydyl enzyme inhibitors did not significantly effect the activity of E. coli K12 peptidase E.

The E. coli K12 peptidase B was however confirmed as a metal-ion dependent activity, stimulated by the presence of both Mn^{2+} and Co^{2+} but inhibited by Zn^{2+} and EDTA. A number of different studies have shown that peptidases D, A, N and T can all be stimulated in their activity by divalent cations, in all cases by Mn^{2+} but for peptidase D, also Co^{2+}, and for peptidase A also Mg^{2+} (Hermsdorf, 1978; Vogt, 1970; Sussman & Gilvarg, 1970; Yang & Somerville, 1976). These same peptidases are all inhibited in activity by the presence of Zn^{2+}, Ca^{2+}, Cu^{2+} and with the exception of peptidase D by Co^{2+}. Peptidase A activity is inhibited by EDTA, peptidase T by another metal-ion chelator O-phenanthroline, and peptidase N activity by the trivalent

cations Fe^{3+}, Cr^{3+} and also EDTA. The evidence therefore demonstrates that these enzymes are metallopeptidases. The observation that peptidase A activity may be inhibited by Co^{2+} whereas peptidases B and D are stimulated in activity by the presence of these same metal ions, combined with the observation of a lack of a detectable metal-ion requirement for peptidase E may indicate different physiological roles for the different peptidases, especially considering their large degree of overlapping substrate specificity. Alternatively the different metal-ion effects may point to a means by which the cell may regulate levels of peptidase activity.

In general the activity of the different E. coli K12 peptidases remains high in a pH range from 7.0-10.0. Peptidase N was found to be active from pH 5.5-10 with optimal activity at pH 8.5 (Yang & Somerville, 1975). Peptidase A showed optimal activity at pH 10.5 with substantial activity recorded at pH 11.0 and much reduced activity at pH 6.0 (Vogt, 1970). The levels of tripeptidase T activity were found to be dependent on both pH and the type of buffer used for pH-activity assays (Sussman & Gilvarg, 1970). In all buffers activity remained high at high pH with 50% tripeptidase activity retained at pH 10 in a Tris-buffer and an optimum pH for activity of 8.5. Substantially lower levels of peptidase T activity were observed in borate buffers with the lowest activity seen in phosphate buffers. The dipeptidase, peptidase D, was reported to have a more narrow range of activity than the other peptidases with activity towards

the abnormal peptide carnosine observed from pH 6.8-7.5 when assays were carried out at 25°C (Kirsch et al., 1978). It seems likely that observable activity may appear at a broader range of pH nearer to the optimum growth temperature of 37°C.

The 2 E. coli K12 aspartyl peptide hydrolysing enzymes partially purified in this work showed differing pH/activity profiles but shared similar pH optima. Peptidase B activity was observed in the range pH 5.0-10 with an optimum activity at pH 7.8. Peptidase E gave optimal activity at approx. pH 7.8 but retained more than 50% optimal activity at pH 10.0 in contrast to peptidase B which showed less than 10% optimal activity at this same pH. The observation of high levels of both peptidase A and E activities at high pH further suggests the possibility of varying cellular controls or roles for different peptidases. However as intracellular pH is strictly regulated in E. coli K12, never reaching as far as it is known, the dizzy heights of pH 10 (Booth, 1985), it may also be the case that such pH profiles are incidental to the essential functions and regulation of these peptidases.

Peptidase A however also appears to be substantially more heat stable than the other peptidases for which data is available. Its enzymatic activity is stable after heating for 3 minutes at 75°C in a 40mM KCl buffer and after heating at 85°C in the same buffer 15% of enzyme activity still remains (Vogt, 1970). From the data in this thesis less than 20% of both

peptidase B and E activity remained after heating for 30 minutes at 50°C. Both of these aspartyl peptide hydrolysing enzymes proved relatively stable after storage for 24 hrs at 4°C or -20°C. Peptidase D was rapidly inactivated at 42°C, and 50% of this enzyme's activity was found to be lost after 24 hrs' incubation at 4°C (Kirsch et al., 1978). Addition of BSA stabilised the activity giving a half life of 1 week for the peptidase when stored in 50% (v/v) glycerol. Peptidase N has been reported to be rapidly inactivated after heating at 70°C (Miller, 1975). Temperature versus stability data is not available in the literature at the present time for either E. coli K12 or S. typhimurium LT2 tripeptidase T. The E. coli K12 aspartyl-peptidases do not appear to have any unusual heat stability especially when compared with peptidase A which, with its multimeric high molecular mass structure, its unusually high activity at high pH and its high thermal stability seems significantly different from the other peptidases.

E. coli K12 peptidases B and E were shown in this thesis to give different responses to various standard enzyme inhibitors. Whereas peptidase E activity appeared to be stimulated to a small degree by the inclusion of 1mM EDTA in the assay medium, peptidase B activity was significantly inhibited at the same EDTA concentration. The serine protease inhibitor PMSF caused a slight reduction in peptidase E activity whereas the sulphydryl enzyme inhibitor PCMB had no effect. Peptidase B activity was inhibited to a low degree by both PCMB and PMSF. Data available

on the effect of sulphydryl and serine protease inhibitors on other E. coli K12 peptidases under discussion here is restricted to studies on peptidase N and peptidase T. Contrary conclusions have been reached on the role of sulphydryl groups in peptidase N activity. In one study, peptidase N was reported to be relatively resistant to inactivation or inhibition by sulphydryl reagents, (Yang & Somerville, 1976). In enzyme assay mixtures, inhibition by sulphydryl inhibitors, N-ethylmaleimide, and p-chloromercuribenzoate only became significant when the inhibitor concentrations exceeded 5mM. The addition of the reducing agent β-mercaptoethanol to these inhibited assay mixtures resulted in restoration of most of the lost activity. These workers concluded that no highly reactive sulphydryl group was connected to the peptidase N active centre. In a later study it was demonstrated that reaction with the sulphydryl agent 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) caused a 50% loss of peptidase N activity at a concentration of less than 0.1mM, similar to the effect seen for the sulphydryl protease papain (McAman & Villarejo, 1982). It was also demonstrated that an 80% loss of peptidase N activity occurred after incubation with 0.6mM N-ethylmaleimide for 5 minutes. These workers concluded that peptidase N has a sulphydryl group essential for activity. Both of these teams of researchers found no significant inhibition of peptidase N activity deriving from the serine protease inhibitors PMSF. Tripeptidase T activity was also not inhibited by PMSF, and no inhibition was observed with soybean trypsin inhibitor (another serine protease inhibitor) or iodoacetate. All of the

different peptidases A, B, D, N, T have been shown to be stimulated in their activity by certain metal ions and inhibited by metal chelating agent. Peptidase N may require a sulphydryl group for activity as well as a metal-ion co-factor. The mechanism of E. coli K12 peptidase E activity and the possible inhibitors affecting this aspartyl-peptidase activity requires further investigation using other inhibitors, for example the metal-ion chelating agent O-phenanthroline, and the sulphydyl agent DTNB, as well as the inclusion of inhibitors used in this work at higher concentrations in assay mixtures.

E. coli K12 peptidase N has been subject to detailed kinetic studies (Yang & Somerville 1976; McAman & Villarejo 1982). This amino acid napthylamide hydrolysing enzyme was determined under specified conditions to have a K_m of 0.185 mM for the substrate alanine-β-napthylamide and a theoretical V_{max} of 4.65×10^{-3} units, giving a turnover number of 4×10^6. The actual maximum turnover number value for ala-β-napthylamide hydrolysis was found to be 2×10^6 moles of product per minute due to product inhibition. Ala-β-NA hydrolysis was found to be inhibited by other amino acid napthylamides which were not substrates for peptidase N leucyl-β-napthylamide this inhibition was competitive, in the case of valyl-β-napthylamide, this inhibition was non-competitive and allosteric in nature. This data suggested the presence of distinct binding sites for these inhibitors on the peptidase surface. Also of interest was the identification of an undefined E. coli K12 peptidase N inhibitory

factor in crude cell extracts. No evidence for any equivalent aspartyl-peptidase activity inhibiting factor was obtained in this thesis.

Km values for the hydrolysis of a number of different amino-acid nitroanilides by peptidase N, ranged from 0.1-0.8 mM under specified conditions with V_{max} ranging from 6.6-33 µmole min^{-1} (McAman & Villarejo, 1982). L-amino acids were found to substantially inhibit peptidase N activity with 2 mM glycine, leucine, proline, aspartate and asparagine giving >97% inhibition under appropriate conditions. It is not known whether this inhibition is of any physiological significance.

A K_m value of 1.4 mM has been determined for the E. coli K12 peptidase A catalysed hydrolysis of Met-Ala-Ser (Vogt, 1970) whereas K_m for the E. coli K12 peptidase T catalysed hydrolysis of Met-Gly-Gly was 3.5 mM, V_{max} 45 µmoles min^{-1} (Sussman & Gilvarg, 1970). K_m values for the peptidase D catalysed hydrolysis of carnosine, Leu-Gly and Gly-Leu were determined at 0.25 mM, 1.5 mM and 0.7 mM respectively (Kirsch et al., 1978).

In common with almost all other peptidases described, the A. calcoaceticus ANA hydrolysing peptidase was shown to be a metallopeptidase inhibited in activity by metal-ion chelating agents. Like the E. coli K12 peptidases B and E, this enzyme hydrolysed a broad range of L-α-aspartyl peptide substrates but as with the E. coli K12 aspartyl peptidases, C-terminal aspartyl-

peptides were not substrates. The ANA hydrolysing peptidase retained high levels of activity at pH10 in common with the E. coli K12 peptidases A and E. The molecular mass of the peptidase however was substantially higher at 460kd than those of the E. coli K12 peptidases although a bacterial peptidase of similarly high molecular mass has been identified in B. stearothermophilus, the 400kd thermophilic aminopeptidase I (Stoll et al., 1972).

The E. coli K12 peptidases and microbial peptidases as a whole vary substantially in molecular mass and physical structure. With the notable exceptions of the E. coli K12 and S. typhimurium LT2 peptidase E enzymes, all of these microbial peptidases appear to have a metal ion requirement for activity. All of the E. coli K12 peptidases are synthesised constitutively but other regulatory data in the literature on the E. coli K12 peptidases is contradictory. Despite this fact it appears that these E. coli K12 peptidase may be under the influence of several global regulatory networks.

7.2 The occurrence, origins and diversity of microbial peptidases

A number of different enzymes occurring in various organisms have been grouped into enzyme families on the basis of similarities in structural and catalytic properties. This section looks at the occurrence and variety of peptidase enzymes and peptide degrading systems, in particular in microorganisms,

with a view to considering the possible evolutionary and enzyme family relationships between the peptidase enzymes.

Data presented in chapter 3 of this thesis demonstrates the widespread occurrence in microorganism of peptidases capable of hydrolysing L-α-aspartyl peptides. The observation that aspartyl-peptides could serve as nutritional sources for both bacterial and fungal strains led to the identification of these peptidases in both types of microorganism. Intracellular aspartyl-peptide hydrolysing activities were found in all bacterial species tested including Escherichia coli, Bacillus subtilis, Acinetobacter calcoaceticus, Pseudomonas fluorescens. Micrococcus luteus, as well as all fungal species tested including Neurospora crassa and Aspergillus nidulans. Extracellular aspartyl-peptide hydrolysing activities were identified in 2 fungal isolates originally selected as possessing high extracellular esterase activity. Bacterial strains were screened for extracellular aspartyl-peptide activity and B. subtilis demonstrated to produce an extracellular aspartyl-β-napthylamide hydrolysing enzyme, probably corresponding to the previously described aminopeptidase III (Desmond et al., 1975).

A brief glance at the relevant literature reveals that the widespread occurrence of L-α-aspartyl-peptide hydrolysing enzyme in microorganisms reflects the position with respect to peptidases showing a range of different specificities.

Leucyl-peptide hydrolysing enzymes have been identified in the bacterial strains E. coli K12 (Miller & Schwartz, 1978), Salmonella typhimurium LT2 (Miller & Mackinnon, 1974), B. subtilis (Desmond et al., 1975) and B. stearothermosphilus (Stoll et al., 1972; Roncari et al., 1975) as well as the fungal strains Saccharomyces cerevisiae (Becker et al., 1982) and Neurospora crassa (Wolfinbarger & Mazluf, 1974). Alanyl-peptide hydrolysing peptidases have been identified in the bacterial strains E. coli K12, S. typhimurium LT2, Proteus mirabilis, Psuedomonas acidivorans, Serratia marcescens and Aerobacter aerogenes (Murgier et al., 1976). Moreover the presence of multiple peptidases in both bacterial and fungal strains has been observed in all systems studied. At least 11 different peptidase activities with varying specifities have been identified in the fungal strain Neurospora crassa (Wolfinbarger & Mazluf, 1980) and at least 10 activities with varying but overlapping specificities in E. coli K12 and S. typhimurium LT2 (Miller, 1987).

Multiple aspartyl-peptide hydrolysing activities were demonstrated to be present in all of the fungal strains tested in this thesis and all of the bacterial strains except for a single rhizobium species, as shown by the presence of multiple activity bands after staining of crude cell extracts separated on polyacrylamide gels. 4 aspartyl-peptide hydrolysing activities have been demonstrated previously in S. typhimurium LT2 (Carter & Miller, 1984).

The biological roles of the multiple peptidase systems are not clear, however slight differences in the substrate specificities of the different peptidases may be required for the efficient degradation of the whole range of possible peptides. 4 different leucyl-peptide hydrolysing enzymes have been described in E. coli K12 each with slightly different but overlapping specificities (Miller & Schwartz, 1978; Hermsdorf, 1980; Miller, 1987). One study has led to the suggestion that aspartyl peptides may be toxic to aspartyl-peptidase deficient strains (Carter & Miller, 1984) and so the presence of multiple aspartyl-peptide hydrolysing enzymes might be required for the rapid elimination of this series of toxic peptides.

No reports of peptidase enzymes in the most ancient microbial kingdom, the Archaebacteria, have been discovered during the course of this study. It would have proven an interesting and simple experiment to test whether members of the kingdom possessed peptidase activities, and indeed whether multiple peptidase activities were present. Archaebacteria possess many of the molecular features of both Eubacteria and Eukaryota, including a complex translational apparatus for protein biosynthesis (Woese, 1987). They are complex entities and it seems reasonable to guess that these earliest of all known microbes possess peptidase activities. However a knowledge of these peptidases might provide us with clues as to the ancestry of the 'modern' peptidases.

The numerous microbial peptidases that have been identified appear with a few exceptions to be metallopeptidases, having a requirement for activity of divalent cations. The metal ions Zn^{2+}, Mn^{2+}, Co^{2+} and Mg^{2+} may all act to stimulate different peptidase activities whereas some activities are inhibited by the presence of Zn^{2+} or Co^{2+}. The aspartyl-peptide cleaving peptidase E from both S. typhimurium LT2 and E. coli K12 has so far not been demonstrated as having a metal ion requirement for activity.

Within the constraints of metal-ion requiring mechanisms, a diversity of substrate specificity exists with different microbial peptidases degrading peptides of both different length and different amino acid composition. In E. coli K12, oligopeptidase A hydrolyses tetrapeptides, peptidase T hydrolyses tripeptides, peptidases N, A and B both di- and tripeptides and peptidase D only dipeptides. pH optima for these enzymes all lie at around pH 7.5-8.5. However the range of pH at which these peptidases remain active varies with some retaining activity at high pH.

The similarities in metal-ion requirements and pH optima may reflect a similarity in catalytic mechanisms. However in terms of molecular mass and sub-unit structures the peptidases vary enormously. The E. coli K12 peptidases range in size from the monimeric 35kd peptidase E reported in this work to the hexameric 323kd peptidase A (Vogt, 1970). Similarly the aminopeptidases

from B. stearothermophilus range in size from the dimeric thermolabile aminopeptidase II, M_r 92kd, to the dodecameric thermostable aminopeptidase I, M_r 400kd (Roncari et al., 1975). The wide range of mobilities for aspartyl-peptidases observed after staining of cell extracts separated on PAGE gels in the thesis suggests a broad range of molecular mass values for aspartyl peptidases both in the same organisms and between different microorganisms. Mammalian peptidases have been found to range from 35kd (bovine pancreas carboxypeptidase B) to about 300kd (bovine lens leucine aminopeptidase) with varying numbers of sub-units, and these enzymes are usually glycoproteins (MacDonald & Barrett 1987). The wide divergence of peptidase structures would appear to suggest that the different peptidases may not be closely related either in structural, mechanistic or evolutionary terms.

The most widely studied group of enzymes, the serine protease family, have been assigned to this particular group on the basis of common tertiary structures, amino acid sequence homology, and the presence of similar catalytic mechanisms and enzyme substrate interactions (Ferscht, 1985). The name 'serine' protease derives from the presence in these enzymes of a uniquely reactive serine residue that reacts irreversibly with organophosphates such as diisopropylfluorophosphate. These serine proteases have been identified in bacterial, fungal and mammalian systems and in general these enzymes are considered to have evolved by a mechanism of divergent evolution from a common ancestral protein.

Could the microbial peptidases form a similar enzyme family evolved from a single ancestral peptidase? Six criteria have been proposed for determining whether 2 or more proteins have evolved from a common precursor:

1. DNA sequences are homologous
2. Amino acid sequences are homologous
3. The 3D structures are homologous
4. Enzyme substrate interactions are homologous
5. The catalytic mechanisms are similar
6. The segments of polypeptide chain essential for catalysis are in the same sequence (Matthews et al., 1981).

The DNA and amino acid sequence data has as yet been determined for only a single microbial peptidase, the E. coli K12 peptidase N (Foglino et al., 1987) making comparative studies of sequence data impossible. The gene encoding the E. coli K12 peptidase D has recently been cloned (Klein et al., 1986) and the DNA and amino acid sequences of this peptidase may soon be available enabling analysis of the possible evolutionary relationships between these enzymes. The diversity of molecular mass and sub-unit composition observed between the different microbial peptidases and observed even within the multiple peptidases of a single bacterium such as E. coli K12, would seem to argue however against the evolution of peptidases from a single ancestral protein. It would seem more probable that structurally different

peptidases with significantly different peptide substrates and perhaps also different metal ion requirements, evolved from separate ancestral proteins. It may be more appropriate to consider the microbial peptidases as composed of several different evolutionary enzyme families.

Some evidence to support such an idea comes from the identification of peptidase N-like enzymes in a wide range of different gram-negative bacteria (Murgier et al., 1976). These enzymes all showed approximately the same mobilities on PAGE gels and hydrolysed the chromogenic substrate L-alanine-p-nitroanalide. The similarities observed between these peptidases may represent membership of a family of peptidases. In addition in the work reported in this thesis many microbial strains were shown to carry 2 aspartyl-peptide hydrolysing enzymes of approximate mobilities 0.3 and 0.5 on PAGE gels. These could potentially represent 2 different evolutionary families of aspartyl peptide hydrolysing enzymes. As can be deduced from the 'ancestral' criteria described above, differences in enzyme structure may not necessarily point to separate ancestral proteins. Amino-acyl tRNA synthetases differ widely in structure but there is some evidence that they are closely related despite their structures (Hartley, 1975). Confirmation of the evolutionary relationships between the different peptidases will however only follow the acquisition of appropriate DNA and amino acid sequence data for a broad range of peptidase enzymes.

The apparent non-essential nature of the microbial peptidases, as evidenced by the viability of E. coli K12 and S. typhimurium LT2 strains deficient in a number of peptidase enzymes (Miller & Schwartz, 1978; Miller & Mackinnon, 1974), would provide a background against which rapid evolution of these enzymes could occur. Very small changes in amino acid sequence have been shown to produce alterations in enzyme specificity. The 2 serine proteases elastase and chymotrypsin differ in 2 amino acid residues around the substrate binding pocket, giving chymotrypsin specificity for peptide bonds containing phenylalanyl and tryptophanyl residues whereas elastase hydrolyses peptide bonds containing small hydrophobic residues such as alanine. Small differences in peptidase substrate binding pockets could therefore provide for a range of peptidase substrate specificities.

A further question arises as to the relationship between the development of peptide transport and peptidases. Peptidases have been demonstrated to have a role in the degradation of toxic peptides and cellular turnover of proteins in E. coli K12 (Yen et al., 1980a) so even in the absence of peptide uptake systems these enzymes would serve a function within these cells. Uptake of peptides in the absence of the capacity to hydrolyse these substrates could adversely affect cell functions and possibly lead to cell death. It is tempting therefore to consider that peptidases originated before development of the capacity to take peptides into the cell and use them as nutritional sources. This

argument is supported by the non-inducible nature of the E. coli K12 peptidases, when the bacterium is grown in the presence of peptide containing media (Higgins et al., 1987; McAman et al., 1988; this thesis), which suggests that nutritional utilisation of externally supplied peptides may be a secondary function for these enzymes.

No direct genetic, biochemical or regulatory link has been established between peptide transport systems and peptidases in E. coli K12. Genes encoding peptidase enzymes and peptide transport systems are scattered around the E. coli K12 genome. Levels of the oligopeptide transport system components and the tripeptidase, peptidase T have been shown however to be induced under anaerobic conditions (de Rienhdt et al., 1988) and it may therefore be the case that peptidase enzymes and transport systems form part of a single as yet undefined global regulatory network. The lack of any operon-like regulatory linkage between transport systems and peptidases still points however towards separate development and evolution of characteristics. The observation that the Gram- positive bacteria B. subtilis and B. licheniformis as well as certain fungal strains possess extracellular peptidase activities (Payne, 1978; Wolfinbarger & Mazluf, 1974) generating amino acids for uptake via amino acid transport systems also emphasises the separation of peptide transport and peptidases, with some fungal strains having been identified as unable to take up extracellular peptides without prior hydrolysis.

7.3 Biological routes for the synthesis of Aspartame: Reverse Hydrolysis and other methods

In chapter 7 of this thesis, a number of different conditions were used in an effort to synthesise the dipeptide sweetener aspartame by a process of reverse hydrolysis. Crude bacterial cell extracts containing aspartyl-peptide hydrolysing enzymes or partially purified peptidases were incubated under a range of different conditions with the aspartame precursors, aspartate and phenylalanine methyl ester but under none of the conditions used was synthesis of the sweetener detected. As predicted by other workers (Oyama & Kihara, 1984) although exopeptidases such as the aspartyl-peptide hydrolysing enzymes may provide some theoretical advantage over chemical methods of synthesis, or methods involving endoproteinase catalysed synthesis as no protection of substrates would be required, problems appear to occur in shifting equilibrium towards the synthetic side.

The lack of success in promoting exopeptidase catalysed reverse synthesis of aspartame reported in this thesis does not reule out the possibility that such a synthesis might not be achieved at some future date. The conditions used in synthesis experiments were not exhaustive nor were the complete range of aspartyl-peptide hydrolysing enzymes used in synthesis experiments. It would perhaps have been more advisable to use an extracellular aspartyl-peptidase such as the *B. subtilis* aminopeptidase III, or a heat-stable aspartyl-peptidase from for

example B. stearothermosphilus in this work, as such enzymes would be likely to be more stable in the 'synthesis' environment than those peptidases used. The observation that no aspartame synthesis was observed in either water-misable organic co-solvents (85% (v/v) 1,4,-butanediol or 60%, 80%, 90% (v/v) glycerol), in a biphasic system (75% ethyl acletate: 25% water), or in an aqueous environment at varying pH, indicates that aspartame synthesis by reverse hydrolysis using exopeptidase enzymes, however is not a promising route for either the small scale or large scale synthesis of aspartame.

Aspartame has already been synthesised using a route which combines both biological and chemical processes (Isowa et al., 1979; Oyama et al., 1981; Nakanishi et al., 1985). The endoprotease thermolysin catalyses synthesis of CBZ-aspartame from the precursors CBZ-aspartate and phenylalanine methyl ester, and this CBZ-aspartame undergoes catalytic dehydrogenation to produce aspartame. Although a number of different endoprotease enzymes have been shown to be able to catalyse this synthesis, the process is reported to encounter problems in the isolation and purification of product. This endoprotease catalysed route for synthesis holds out some promise for future large scale production of the sweetener once these technical problems have been overcome.

The implications of the reported synthesis of aspartame in high yields using whole bacterial cells (Ajinomoto, 1987) may be

substantial for the world sweetener market. If technical difficulties have been overcome and a pure sweetener product can be generated in abundance and cheaply as would seem likely from what has been revealed about this synthesis, then the 'whole cell' aspartame synthesis method may become the major method for sweetener synthesis employed worldwide. Sucrose could possibly be replaced by aspartame as the most commonly used sweetener in the western world! The theoretical basis of this synthesis process is not clear. Reverse hydrolysis within the bacterial cell catalysed by peptidase enzymes seems unlikely due to kinetic, energetic, and equilibrium considerations which favour hydrolysis over synthesis. Possibly the normal protein synthesis mechanisms may, by some unknown mechanism be used in dipeptide synthesis. It did not prove possible to synthesise aspartame in the work reported in this thesis following the patented procedures, using bacterial strains from the Trent Polytechnic culture collection.

One other biological approach to the synthesis of the sweetener has involved adaptation of the techniques of genetic engineering (Doel et al., 1980). This approach involved synthesis of 2 dodecacleoxynucleotides of defined sequence which, when polymerised, inserted into an appropriate plasmid, and read in the correct phase encode the repeating dipeptide poly (aspartyl-phenylalanine). Clones of E. coli K12 were isolated encoding up to 150 repeats of aspartyl-phenylalanine. Digests of the polymer using different endoprotease enzymes produced complex

mixtures of peptides, including Phe-Asp and Asp-Phe dependent on the enzyme used, and it was proposed that this method could form the basis of a route for peptide syntheses. No subsequent reports on the use of this method for aspartame synthesis have been published.

Biological routes for the synthesis of aspartame appear to hold significant promise for the large scale manufacture of this sweetener. The use of whole bacterial cells for synthesis would seem to provide the cheapest and most efficient route and could possibly be applied to the synthesis of other peptides. Endoprotease catalysed reverse hydrolysis provides an alternative route which is currently the subject of substantial research.

7.4 Unanswered questions/future work

Some fundamental questions remain unanswered despite the work carried out in this thesis. There is still room for doubt on the question as to whether N-terminal aspartyl-peptide degrading enzymes in addition to peptidases B, E and Q, are synthesised by *E. coli* K12. Although no such activities were observed using the colorimetric activity assay employed in this thesis, the inability of this assay to detect *E. coli* K12 peptidase M (Miller *et al.*, 1987) and other activities including the Phe-Asp activity from the same organism (this thesis) leaves open the possibility of the presence of additional N-terminal aspartyl-peptide hydrolysing activities. If such activities are stable to extraction procedures then their presence can probably

be established using standard separation techniques such as gel filtration and ion-exchange chromatography combined with an effective quantitative peptidase assay.

The mechanism of E. coli K12 peptidase E catalysed hydrolysis of its aspartyl-peptide substrates remains to be defined. No specific metal ion requirement could be demonstrated for this peptidase, providing a parallel with the peptidase E identified in S. typhimurium LT2. As the E. coli K12 peptidase E appears to be neither a sulphydryl enzyme or a serine protease, further inhibitor studies will be required to define its catalytic mechanism, in particular using metal-ion chelating agents, as metal ions have been identified as affecting the activity of many other microbial peptidases. Kinetic studies remain to be performed with both E. coli K12 peptidases B and E and the sub-unit structures of these enzymes have yet to be defined, a process which will require complete purification of both activities.

Now that the map position of the E. coli K12 pepB gene is available this should facilitate the cloning of the peptidase B encoding gene and should enable more detailed studies on the regulation of peptidase B activity. This would also provide a further insight into the role of aspartyl-peptide hydrolysing enzymes in the cell and help establish the presence or absence of regulatory networks affecting expression of all peptidase encoding genes. Much scope remains for regulatory studies with

both *E. coli* K12 peptidases B and E.

Finally, establishment of the evolutionary relationships between different peptidases will require in particular the determination of DNA sequences of peptidase encoding genes and the amino acid sequences of peptidases. Many experiments remain to be carried out before a full understanding of the nature, evolutionary origin and biological role of microbial peptidases can be claimed.

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Form CNAA 10 Decl

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Degree for which thesis is submitted: DOCTOR OF PHILOSOPHY (PhD)

1 Statement of advanced studies undertaken in connection with the programme of research (Regulations 3.8–3.10 refer)

The candidate has attended lectures and seminars held at Nottingham University and Leicester University relating to the relevant research area. He has also attended relevant departmental seminars at Nottingham Polytechnic. A communication relating to the examined work was presented at the 1988 Biochemical Society Meeting held at Nottingham University and the candidate also attended the 1985 Biochemical Society Meeting held at Warwick University. Throughout the course of this work the candidate was familiarised with biochemical and genetic techniques.

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DEDICATION

This thesis is dedicated to my mum , and to my dad who died this Valentines day , February 14th 1990 ; he will be remembered with love and affection by us all.

Identification and characterization of an aspartate-specific peptidase (peptidase E) in *Escherichia coli* K12

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Investigations have been undertaken on the peptidase enzymes involved in the degradation of L-α-aspartyl peptides in *Escherichia coli* K12. This organism produces at least six broad specificity peptidases, denoted N, A, B, D, Q and P which have partially overlapping specificities. These peptidases are thought to be involved in the catabolism of intracellular proteins, hydrolysis of small peptides supplied as nutritional sources and the breakdown of abnormal cell proteins (Miller, 1987). Previous studies on these peptidases by other workers have suggested that only peptidases N, A, B and D are capable of hydrolysing the dipeptide Asp-Leu and a strain lacking these enzymes was reported to be unable to utilize this dipeptide as a source of required leucine (Miller & Schwartz, 1978).

Contrary to these findings, our own investigations have indicated that both *N*-terminal and *C*-terminal aspartyl peptides could serve as sole source of required amino acids for K12 strains carrying not only a full complement of peptidase enzymes but also for strains lacking peptidases N, A, B, D and Q.

Sonicated crude extract from these strains were separated on 7.5% (w/v) non-denaturing polyacrylamide disc gels (Davis, 1964) and gels stained for peptidase activity (Miller & Mackinnon, 1974). With either Asp-Leu or Asp-Phe-OMe as substrate, a single band of peptidase activity (relative mobility 0.5) was observed in peptidase-deficient strains, whereas in wild-type strains an additional band of activity (relative mobility 0.3) corresponding to peptidase B was also found. The activity of the faster running peptidase was not present when Leu-Gly, Phe-Asp and Glu-Phe were used as assay substrates, nor was its Asp-Xaa-degrading activity visibly affected by performing assays in the presence of metal ions or EDTA.

This newly found peptidase activity was purified in a three-step procedure using ammonium sulphate fractionation, gel filtration and ion-exchange chromatography. The enzyme eluted from a Sephadex G-200 gel filtration column at a volume corresponding to an M_r of 35 000 based on the elution of molecular mass standards. This M_r value was confirmed by plotting the retardation coefficients from different concentration polyacrylamide gels against the M_r for both the peptidase enzyme and molecular mass standards (Hedrik & Smith, 1968).

The 35 000 value for M_r corresponds to that of an aspartate-specific peptidase, peptidase E, previously identified in *Salmonella typhimurium* (Carter & Miller, 1984). Owing to this observation and other similarities between the two enzymes, both in substrate specificity and other biochemical properties, the K12 peptidase described here was designated peptidase E.

As a means of assessing in more detail the substrate specificity of the K12 peptidase E, the semi-purified enzyme was incubated with different aspartyl-peptide substrates. Reaction products were derivatized with *o*-pthalaldehyde/2-mercaptoethanol and analysed by reversed phase h.p.l.c. (Griffin *et al.*, 1982). Relative rates for the hydrolysis of different peptides are shown in Table 1. Comparison of this K12 peptidase E data with data for the *S. typhimurium* enzyme (Carter & Miller, 1984) shows strong similarities in substrate specificities. Neither enzyme hydrolyses Asp-β-napthylamide, Leu-Gly or CBZ-Asp and both show high levels of peptide hydrolysis with Asp-Ala and Asp-Lys as substrates, although there is a substantial difference between the two enzymes in hydrolysis rates with the substrate Asp-Phe.

Table 1. *Substrate specificity of peptidase E*

*Peptides at a final concentration of 3 mM were incubated in 0.1 M-Tris/HCl (pH 7.5), 1 mM-EDTA at 37°C for 30 min with purified enzyme (0.24 mg of protein/ml). Following derivatization with *o*-phthaladehyde/2-mercaptoethanol, reaction products from incubation mixtures were analysed by reversed phase h.p.l.c. The rate of hydrolysis is expressed relative to that of Asp-Leu (as 100 %). Asp-Leu was hydrolysed at a rate of 1 μmol/min per mg of protein.

Substrates	Relative hydrolysis*
Asp-Leu	100
Asp-Ala	165
Asp-Phe	60
Asp-Val	105
Asp-Tyr	83
Asp-Phe-OMe	27
Asp-Lys	140
Asp-Gly	54
Asp-Glu	57
Not hydrolysed	
Asp-β-napthylamide	
CBZ-Asp	
Leu-Gly	

These data therefore indicate that contrary to suggestions from previous data there exists in *E. coli* K12 an aspartate-specific peptidase with properties very similar to those of the aspartate-specific peptidase E from *S. typhimurium*. The K12 peptidase B enzyme is shown to hydrolyse aspartyl-peptide substrates, and so it appears that there are at least two aspartyl-peptide degrading enzymes in *E. coli* K12.

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DNA–protamine interactions studied by dielectric and optical measurements

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Nucleoprotamine is a highly condensed complex between DNA and protamine, a small arginine-rich protein, that occurs in the nuclei of sperm cells of several animal species. X-ray diffraction studies on nucleoprotamine and on complexes reconstituted from purified DNA and protamine have shown that protamine is wrapped around the DNA (Fita *et al.*, 1983). It is still uncertain, though, whether the protamine molecule is preferentially (or exclusively) located in the DNA minor or major groove. By dielectric and optical measurements we attempted to gain information on both the fate of bound water molecules and on which of the DNA grooves is primarily involved in the establishment of complexes *in vitro* between herring sperm DNA and herring sperm protamine (clupeine).

We performed the measurements of the complex dielectric constant by a cavity perturbation method at 10 GHz in the temperature interval from −20 to +45°C, and analysed the experimental data in terms of a three component system (solute, free water and bound water) fitted with a suitable mixture formula (Kraszewski *et al.*, 1976). Details on the experimental equipment and on the elaboration of the data are reported elsewhere (Bonincontro *et al.*, 1986). By this technique a specific hydration of 35 water molecules per nucleotide can be measured for DNA. A value of 3 water molecules per amino acid residue was found for protamine solutions, to be compared with the number of 12 in the case of free arginine; a difference which is consistent with a folded conformation of the protein in the absence of DNA. On the other hand, it is known by theoretical computation (Clementi & Corongiu, 1982) that almost 60% of the DNA hydration shell is made of water molecules lying in the grooves, loosely bound and easily excluded upon interaction of the polyion with other macromolecules. We therefore measured the complex dielectric constant of the DNA–clupeine complex prepared according to a reconstitution method (Willmitzer *et al.*, 1977) by mixing 20 mM-DNA (nucleotide) and clupeine (amino acid; chloride form) solutions at the desired ratios in 1 M-NaCl, 50 mM-Tris/HCl, pH 7.5, and lowering the ionic strength down to 0.3 M-NaCl by dropwise addition of distilled water. The complexes were recovered by centrifugation, resuspended and checked spectrophotometrically for DNA content after alkaline hydrolysis (Ohba & Hayashi, 1972), and for arginine content after acid hydrolysis and a colorimetric reaction (Izumi, 1965). From the dielectric measurements on the reconstituted complexes we were able to calculate a value of 11 bound water molecules per solute molecule (we cannot distinguish between nucleotide and amino acid), which is less than half the value expected for the uncomplexed components.

We analysed the reconstituted complexes also for their ability to inhibit the binding to DNA of actinomycin D (ACTD), which is known to intercalate via the minor groove (Jain & Sobell, 1972). The binding isotherms are reported in Fig. 1, together with a description of the method employed. No binding of ACTD to the protein was detected in control samples. An excluded-site model (McGhee & Von Hippel, 1974) was used to compute the binding parameters. As far as the strong mode of binding is concerned, the intrinsic association constant of ACTD to DNA is found unchanged and equal to 2.5×10^{-5} M^{-1} in all the samples, while the apparent number of available sites on the DNA decreases to 87% and 79% when the clupeine to DNA molar ratios are 0.1 and 0.3, respectively. These results fit the model for non-competitive binding (Le Pecq & Paoletti, 1967), suggesting that clupeine is not located in the same groove as ACTD (i.e. the minor groove) under the present experimental conditions.

Abbreviation used: ACTD, actinomycin D.

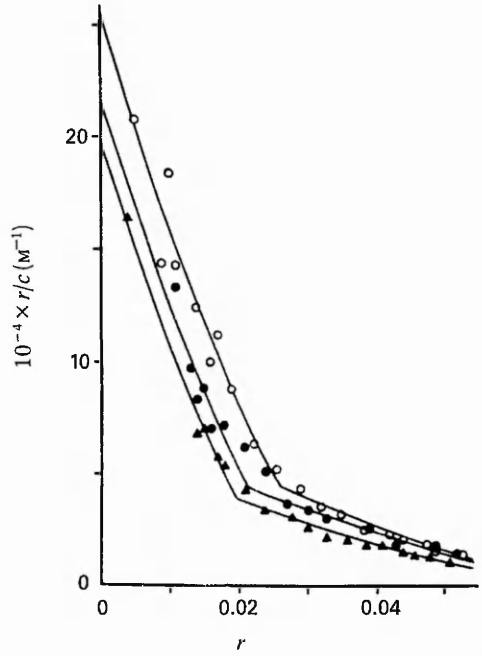

Fig. 1. *Scatchard plots of the binding of ACTD to DNA (○) and to DNA–clupeine complexes at clupeine to DNA molar ratios of 0.1 (●) and 0.3 (▲)**

The complexes were obtained as very diluted solutions (0.3 mM-DNA) in 0.5 M-NaCl. Small aliquots of a concentrated ACTD solution were added to a fixed amount of the polymer in 4 cm quartz cells and absorption readings at 440 nm were taken with an LKB Ultrospec II spectrophotometer. r, Bound ACTD molecules per nucleotide; c, molar concentration of free ACTD.

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