PEPTIDE METABOLISM IN THE LACTOCOCCI AND ITS REGULATION

A thesis presented in partial fulfilment of the requirements for the degree of Master of Science in Biochemistry at Massey University

IAN LINTON MOORE

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Abstract

Aspects of peptide metabolism in the lactococci have been investigated to increase the understanding of how these nutritionally fastidious bacteria, which have a central role in the dairy manufacturing industry, are able to grow in a complex medium such as milk.

Peptide metabolism by lactococci in milk encompasses the processes by which large oligopeptides, produced from milk-caseins by the extracellular activity of the cell wall-associated proteinase, are converted into an intracellular pool of metabolisable amino acids. This involves the activities of both membrane-bound transport systems and peptidases.

Early research into lactococcal peptide utilisation has proposed significant differences between *Lactococcus lactis* strains with respect to the mechanisms by which these bacteria utilise peptides in their environment. More recent studies of the lactococcal peptide carrier systems, based on intensive studies of only a single strain, have proposed a major role for a carrier system capable of transporting di- and tripeptides, and a subsidiary role for another system transporting oligopeptides containing four or more residues. Yet to date, peptidases with an extracellular location capable of degrading the large casein-peptides into smaller peptides have not been isolated.

This current study has attempted to investigate more fully the *in vivo* activity of the oligopeptide transport system, and to assess whether it may have a more fundamental role in peptide utilisation than previous work has suggested. For this study a model series of homologous peptides of increasing size from the dipeptide Val-Gly to the octapeptide Val-Gly_7, all based on the essential amino acid valine, was used. The larger peptides in this series, Val-Gly_3, Val-Gly_4 and Val-Gly_7, were synthesised for this work. The ability of *Lactococcus lactis* subsp. *cremoris* E8 to transport these peptides, and to grow in a chemically defined medium where they constitute the sole source of the essential amino acid valine, was studied . Preliminary peptide uptake studies were also performed using oligopeptides derived from a proteolytic cleavage of β-casein. The collective results of these studies suggest that the upper size limit, and the relative activity of this transport system, may be sufficient to permit this strain to utilise relatively large casein-derived oligopeptides without the need for hydrolysis into smaller peptides and free amino acids.

A comparative study of peptide transport by a number of *Lactococcus lactis* strains was undertaken to investigate previously published observations indicating significant differences in the mechanisms of peptide uptake between lactococcal strains.

While the results of this comparative study are consistent with the general model proposing two separate peptide carrier systems, they have revealed that significant differences can exist between strains in the relative activities and possible substrate specificities of these transport systems consistent with previous work that the lactococci have only two peptide carrier systems. These observations imply the need for caution in extrapolating the results obtained from the study of a single strain to lactococci as a whole. In contrast to the finding of significant strain differences with respect to the relative rates of peptide transport, a comparative study of the relative activity of six different intracellular peptidases showed relatively few differences in peptidase activity between strains.

An investigation was also carried out to assess whether the peptidases and transport systems involved in the utilisation of peptides were nutritionally regulated. No clear evidence was obtained for the significant induction of either the intracellular peptidase complement or the di-/tripeptide transport system.

An attempt was also made to isolate a mutant of *Lactococcus lactis* subsp. *cremoris* E8 unable to utilise dipeptides, to assess whether the di-/tripeptide transport system or the intracellular dipeptidase of this strain were essential to casein utilisation. This attempt was not successful.

Acknowledgements

The acknowledgements section of most theses is usually one of the last things to be completed, and can often be glossed over in the rush to meet submission dates. Yet in many ways this is probably one of the more important aspects of a thesis, as without the advice, encouragement and support of supervisors, colleagues, family and friends few research projects would ever reach the written stage. Therefore, while I will be taking credit for the work presented in this thesis, I would like to acknowledge the contributions of the following people.

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Ian Moore December 1994.

List of Abbreviations

AβClA	L-alanyl-β-chloroalanine
ATP	adenosine triphosphate
β-CDM	β-casein defined medium
Boc	tertiary-butyloxycarbonyl
CDM	chemically defined medium
CHM	casein hydrolysate medium
CE	capillary electrophoresis
DIEA	di-isopropyl ethylamine
DCC	dicyclohexylcarbodimide
DCU	dicyclohexylurea
EtOH	ethanol
FITC	fluorescent isothiocyanate
Fmoc	9-fluorenylmethoxycarbonyl
HPLC	high performance liquid chromatography
SIMS	secondary ion mass spectrometry
SMM	skim milk medium
SPPS	solid phase peptide synthesis
STB	screw top bottle
TCA	trichloroacetic acid
TFA	trifluoroacetic acid
TMG	1,1,3,3-tetramethylguanidine
UBM	undefined broth medium.
Val-Gly	Valyl-glycine.
$Val\text{-}Gly_2$	Valyl-glycyl-glycine.
$Val\text{-}Gly_3$	Valyl-glycyl-glycyl-glycine.
$Val\text{-}Gly_4$	$Valyl\text{-}(glycyl)_3$-glycine.
$Val\text{-}Gly_7$	$Valyl\text{-}(glycyl)_6$-glycine.
$Val\text{-}Gly_9$	$Valyl\text{-}(glycyl)_8$-glycine.

Abbreviations used for amino acids:

amino acid	three letter abbreviation	one letter abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tyrosine	Tyr	Y
Tryptophan	Trp	W
Valine	Val	V

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Chapter One: Introduction

1.1 The importance of lactic acid starter bacteria in the dairy industry.

The conversion of lactose to lactic acid, and the degradation of casein micelles to produce a heterogeneous mixture of peptides and amino acids, are key biochemical events in the manufacture of fermented milk products such as yoghurt and cheese.

Of the various lactic acid bacteria capable of carrying out these enzymatic processes, it is members of the genus *Lactococcus*, particularly *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* that are employed preferentially in industry as starter cultures. (In future these subspecies will be referred to as *L. lactis* and *L. cremoris* respectively).

All lactococci are strictly fermentative, obtaining their ATP and reducing equivalents from the glycolytic metabolism of lactose to lactic acid. The production of lactate acidifies the milk to a final pH of 4 to 5. This acidification facilitates casein coagulation by dissolution of colloidal calcium phosphate in the micelles, and prevents the growth of undesirable micro-organisms in the resulting product.

Lactococcal bacteria have complex nutritional requirements. In addition to vitamins and nucleotides, they are unable to synthesise many of the amino acids required for growth (Reiter and Oram, 1962). *L. lactis* strains require fewer essential amino acids than *L. cremoris* strains (Smid, 1991).

Lactococci can exhibit exponential growth in milk, with a doubling time of 60 minutes at 30°C. Growth to high numbers over a short period is crucial to getting acid production at levels needed to form milk curds on a commercial scale (Laan *et al.*, 1989). Milk however, is a suboptimal medium for these fastidious bacteria, levels of free amino acids and usable peptides being well below their minimal requirement for protein synthesis (Thomas and Pritchard, 1987). More than 80 percent of milk nitrogen is locked up in the proteins α_{s1}, α_{s2}, β and κ-caseins.

The ability of lactococci to grow to high densities in milk is only possible because of a complex proteolytic system which enables them to rapidly degrade these caseins into metabolisable peptides and free amino acids.

1.2 The proteolytic system of lactococcal starter bacteria

The three essential components of the proteolytic system of starter bacteria are the proteinases, the peptidases and the transport systems.

1.2.1 Proteinases

These are cell wall associated serine proteases which catalyse the first step in milk protein degradation, namely the extracellular hydrolysis of caseins into oligopeptides of varying length.
In general these enzymes are plasmid encoded, show an optimum activity at pH 6.6, and have a size range of 80- 180 kDa depending on the method by which they are isolated from the cell (Pritchard and Coolbear, 1993).

The genetic determinants of these proteins are now well characterised. A pre-pro protein encoded for by the *prtP* gene undergoes post-translational modifications in association with a *prtM* gene product before insertion into the cell membrane. Sequence analysis of the *prtP* genes encoding proteinases from different lactococcal subspecies show them to be highly conserved (Kok, 1990, 1991).

On the basis of the specificity of casein hydrolysis, two types of proteinase are currently recognised (Exterkate and de Veer, 1989; Visser *et al.*, 1986). Details of these are summarised in Table 1.2.1.1

Table 1.2.1.1: Classification of proteinases based on their activities towards caseins.

PROTEINASE TYPE	SPECIFICITY	STRAINS
P_1 (HP type)	Major activity towards β–casein and minor activity towards κ-casein. No activity towards α_{s1}-casein	*L. cremoris* HP, WG_2 E_8* *L. lactis* NCDO606 NCDO607
P_{111} (AM_1 type)	Activity towards α_{s1}, β and κ-caseins	L. cremoris SK_{11}, AM_1 E_8*

* NB. E_8 shows a mixture of both proteinase types and so may represent an intermediate form.

These proteinase types differ in their time dependent-cleavage patterns of β and κ-caseins (Reid *et al.*, 1991a; 1994), as summarised in Figure 1.2.1.1.

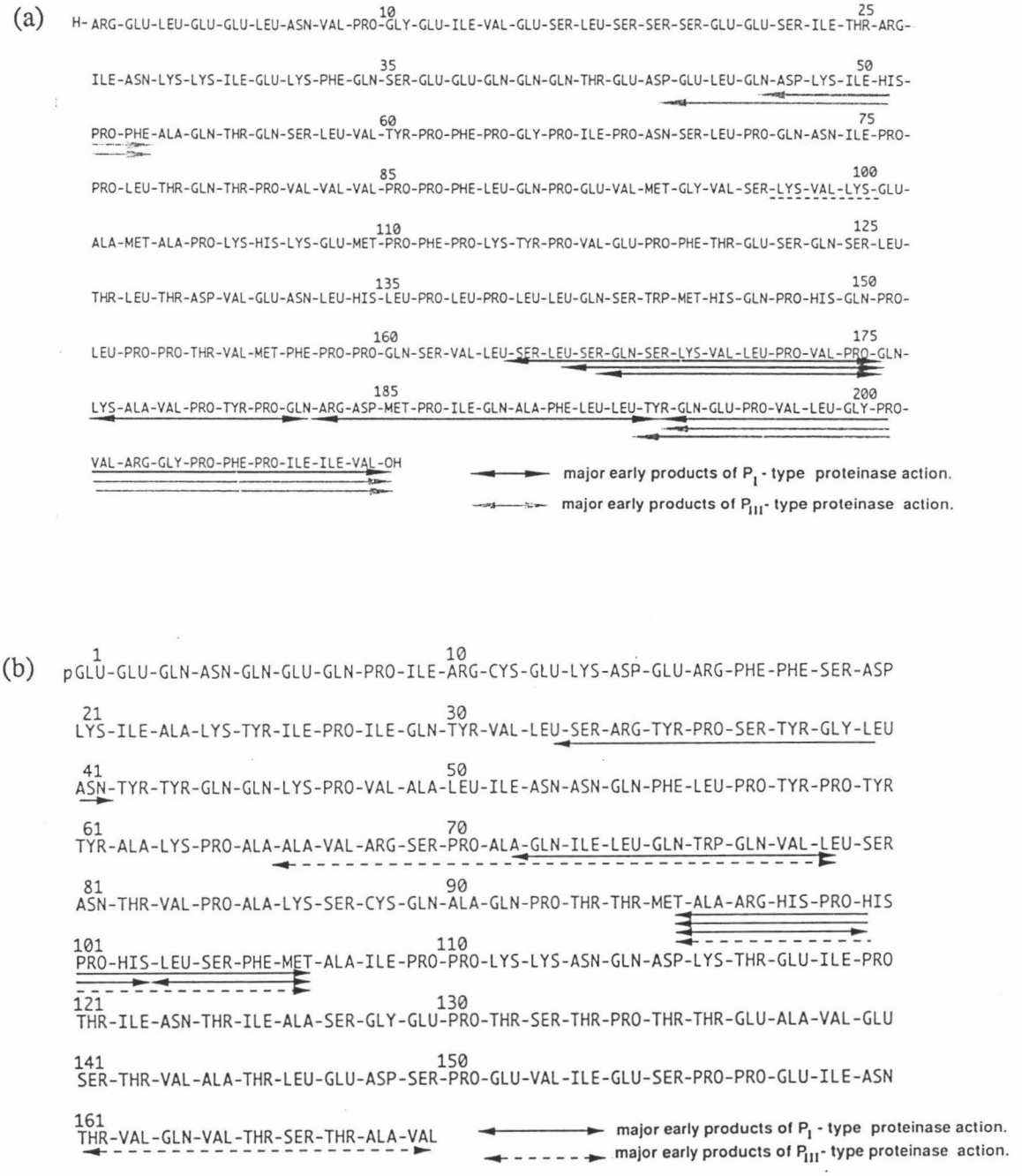

Figure 1.2.1.1 Major peptide products produced by digestion of (a) β–casein (b) κ-casein by the cell envelope-associated proteinase from *L. cremoris* H2 (a P_1-type proteinase-producing strain) and *L. cremoris* SK11 (a P_{111}-type proteinase producing strain).

During growth in milk, the lactococcal cells will be supplied with different oligopeptide products depending on whether they have a P_I or P_{III} type proteinase.

Proteinase synthesis is regulated by both calcium levels in the medium and the nature of the nitrogen source upon which the cells grow. Calcium increases the level of proteinase activity while high levels of free amino acids or small peptides act instead to inhibit proteinase synthesis (Exterkate, 1985).

Recent studies on proteinase gene expression in genetically manipulated strains of lactococci containing multiple copies of the *prtP gene*, suggests that there are differences between strains in the stringency with which this gene is regulated (Bruinenburg *et al.*, 1992).Work with proteinase negative strains has shown these enzymes are essential to growth of lactococcal bacteria in milk.

1.2.2. Peptidases

Lactococci possess a large array of enzymes capable of degrading the oligopeptide products produced by the action of the cell wall-associated proteinase. A list of the peptidases isolated to date are presented in Table 1.2.2.1. While most of these enzymes have now been well characterised *in vitro*, several important questions remain unanswered about their activity *in vivo*.

One such question concerns the regulation of peptidase activity in response to differences in the composition of the growth medium. Law (1977) found that dipeptidase levels in *lactis* strains were lower when the cells were grown on amino acid-defined media than in yeast broth, suggesting that the presence of peptides leads to an increase in the activity of this peptidase.
However, van Boven *et al.* (1988) reported no observable differences in dipeptidase activity of the *lactis* strain Wg_2 when grown on different nitrogen sources. A comprehensive study is required to resolve these differences.

Another unresolved question is which of the various peptidase activities listed in Table 1.2 2.1 are essential for growth upon milk caseins. Answers to this question are best approached by the isolation of mutants deficient in a particular peptidase activity. Already such work using the highly specific technique of integrative gene inactivation (Leenhouts *et al.*, 1991) has demonstrated that neither endopeptidase PepO (Mierau *et al.*, 1993) nor X-prolyl dipeptidyl aminopeptidase (Mayo *et al.*, 1993) is essential for growth of *lactococci* in milk. Eventual identification of the essential enzyme complement for growth on casein is important for the development of improved industrial starters.

Table 1.2.2.1 A summary of the lactococcal peptidases characterised to date.

ENZYME	SPECIFICITY	REFERENCES
Dipeptidase	X---Y	Hwang *et al.*, 1981;1982. van Boven *et al.*, 1988
Tripeptidase (PepT).	X---YZ	Bosman *et al.*, 1990.
Endopeptidase LEPI LEPII PepO	...WX---YZ...	Yan *et al.*, 1987a Yan *et al.*, 1987b Tan *et al.*, 1991
Aminopeptidase A (PepA).	Glu---XY... Asp---XY...	Exterkate *et al.*, 1987 Niven, 1991
Aminopeptidase N (PepN)	W---XY...	Tan and Konings, 1990. Exterkate *et al.*, 1992 Tan *et al* ., 1992 Midwinter and Pritchard., 1994
Aminopeptidase C (PepC).	W---XY...	Neviani *et al* ., 1989 Chapot-Chartier *et al.*, 1992
Prolidase	X---Pro	Kaminogawa *et al.*, 1984 Booth *et al.*, 1990
Proiminopeptidase	Pro---X	Baankreis *et al.*, 1991 Zevaco *et al.*, 1990
X-prolyl dipeptidyl aminopeptidase	X---ProY	Keifer-Partsch *et al.*, 1989 Lloyd and Pritchard, 1991

Much debate also surrounds the cellular location of the peptidases, as will be discussed in Section 1.5.

1.2.3 Transport proteins

Research to date has identified three possible routes by which the products of extracellular protein and peptide degradation could enter the cell, namely as free amino acids, as di- or tripeptides, or as oligopeptides.

(i) Amino acid transporters.

These are integral membrane proteins which are highly selective for specific structural types of amino acids. Different bioenergetic mechanisms are employed in their function (Konings *et al.*, 1989).

(a) proton motive force linked transporters.

Couple transport of an amino acid to the movement of protons down their concentration gradient.
Examples: Val, Leu, and Ile by the branched chain amino acid transporter.
Ala, Gly and Ser by the neutral amino acid transporter

(b) antiporters/ exchange transporters

Couple the outward movement of a product down its concentration gradient with the inward movement of a substrate.
Example: arginine- ornithine antiporter in *L. lactis* strains.

(c) phosphate bond linked transporters

Couple amino acid transport directly to the hydrolysis of ATP or some other "high energy" phosphate bond.
Example: glutamate transport

As yet no genes for these transporters have been identified or characterised .

Expression of some amino acid transport systems appears to be regulated by peptides in the growth media (Poolman and Konings, 1988; Smid, 1991).
The arginine-ornithine antiporter has also been shown to be repressed when cells are grown in arginine-deficient media (Konings *et al.*, 1989).

(ii) Di-/tripeptide transport

The failure of tripeptides to inhibit Glycyl[^{14}C]leucine uptake in early studies with the *cremoris* strains E8 and 2016 suggested that separate proteins transported dipeptides and tripeptides (Law, 1978)

However it is now believed that one major system transports both peptide types. Strong evidence supporting this conclusion has come from the isolation of a mutant of the *cremoris* * strain ML_3 resistant to the action of the toxic dipeptide L-alanyl-β-chloroalanine (AβClA). When cleaved, this peptide releases β-chloroalanine which blocks an alanine racemase essential to cell wall formation. Lactococci which can grow in its presence are deficient in their ability to either transport this peptide, or to hydrolyse it.
Careful physiological characterisation of this mutant showed it to be deficient in its ability to transport both dipeptides and tripeptides.
Tripeptide transport mutants resistant to the toxic tripeptide β-chloro-L-alanyl-L-alanyl-L-alanine are phenotypically identical to the AβClA mutants (Smid, 1991).

Competition studies with these mutants suggests that this transport protein has a broad substrate specificity, except for di- or tripeptides containing arginine. Free amino acids and oligopeptides are not transported by this protein.

Transport by this system is believed to be a two step process, peptide translocation across the cytoplasmic membrane by an integral membrane protein being followed by intracellular hydrolysis by peptidases (Smid *et al.*, 1989a).

Studies of membranes from the wild type ML3 strain fused with liposomes containing beef heart cytochrome c oxidase has shown transport to be an active process driven by the proton motive force (Smid *et al.*, 1989a).

The inability of di-/tripeptide transport mutants to grow in media where β-casein is the sole nitrogen source demonstrates that one or more essential amino acids from this milk protein must enter the cell only as a dipeptide or tripeptide. (Smid *et al.*, 1989b).

Di-/tripeptide transport is also important to meeting the proline demands of a rapidly growing starter culture. This amino acid makes up over 18 percent of β-casein

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* ML3 was formerly regarded as a *lactis* strain. It now has been shown to be much more closely related to the *cremoris* strains (Godan et al., 1992).

It is essential to *L. cremoris* strains for growth, and stimulatory to *L. lactis* strains. There is no carrier protein for free proline and although passive diffusion does occur it does so at very slow rates. Proline containing peptides are however good substrates for this peptide transport system (Smid *et al.*, 1990).

Work with the *cremoris* strain E_8 grown on different nitrogen sources suggests that this transport system is constitutively expressed (van Boven *et al.*, 1988).

Recently the gene encoding this protein, *dtpT*, has been cloned and sequenced (Hagting *et al.*, 1994). The *dtpT* gene encodes for a 463 residue protein , which on the basis of topology studies, is believed to be composed of twelve membrane spanning helices (see Figure 1.2.3.1).

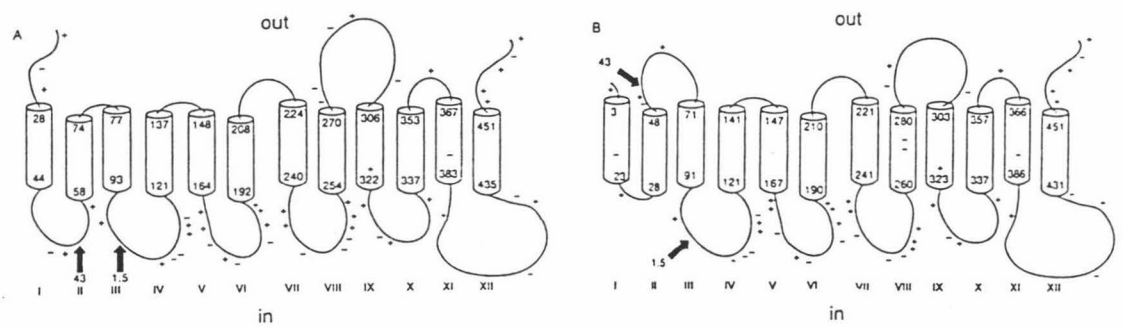


Figure 1.2.3.1 Possible topological models of the di-/tripeptide transport protein of *Lactococcus lactis* (Hagting *et al.*, 1994).

This protein has no significant homology with other bacterial peptide transport systems and so may represent a new type of transport protein.
Future work with the DtpT protein should provide clearer answers with respect to the mechanisms of transport and regulation of that process.

(iii) Oligopeptide transport

Various research supports the presence of a membrane carrier system for oligopeptides. The early work of Rice *et al.* (1978), and Law (1978) showed that oligopeptides can be used as an amino acid source. Apart from the results of

competition studies, little information was obtained about the mechanism by which such peptides were utilised.
For example, it was not known whether oligopeptides greater than three residues were transported intact , or only after hydrolysis into di -/tripeptides and amino acids

The isolation of *L. cremoris* ML_3 mutants deficient in L-alanine, di-L-alanine and tri-L-alanine transport, but still capable of accumulating alanine when it was supplied as a tetrapeptide or pentapeptide suggested strongly that a separate transport system for oligopeptides did exist (Kunji *et al.*, 1993).

These results are supported by complementation studies. The introduction of a cloned DNA fragment from *L. lactis* strain SSL135 into a proteinase-positive strain not capable of growth in milk , enabled this mutant to grow on tryptic peptides of casein (Tynkkynen *et al.*, 1993). Work with the DNA fragment in *Escherischia coli* cells showed that it encoded an oligopeptide transport system (Tynkkynen *et al.*, 1993).

Whereas the di-/tripeptide transport system is encoded for by one gene, the oligopeptide transport system appears to be encoded for by an operon of five genes - *opp* DFBCA (Tynkkynen *et al.*, 1993). The proteins produced from the *opp* DFBCA operon constitute a system that is characteristic of the ATP-binding cassette family of transport proteins (see Figure 1.2.3.2). Oligopeptide transport is an active process which occurs in the absence of electrochemical gradients and can be inhibited by ATPase inhibitors such as vanadate. These results suggest that transport is coupled to hydrolysis of ATP (Kunji *et al.*, 1993).

Work with *cremoris* ML_3 mutants suggests that this protein has a narrow substrate specificity. Oligopeptides containing prolyl, glutamyl, aspartyl or arginyl residues are not substrates for this transport protein (Smid, 1991).

Tetraalanine uptake in these mutants was also found to occur at a much slower rate than dialanyl or trialanyl uptake, indicating this system to be significantly less active than the di-tripeptide transport system. These results are supported by the earlier studies of Rice *et al.* (1978). They found that rates of $Gly_3[^{14}C]$Leu-Gly and $Gly_2[^{14}C]$Leu-Gly uptake by the cremoris strain C_{10} to be one-seventh and one-fifth the rate of Gly-$[^{14}C]$Leu uptake.

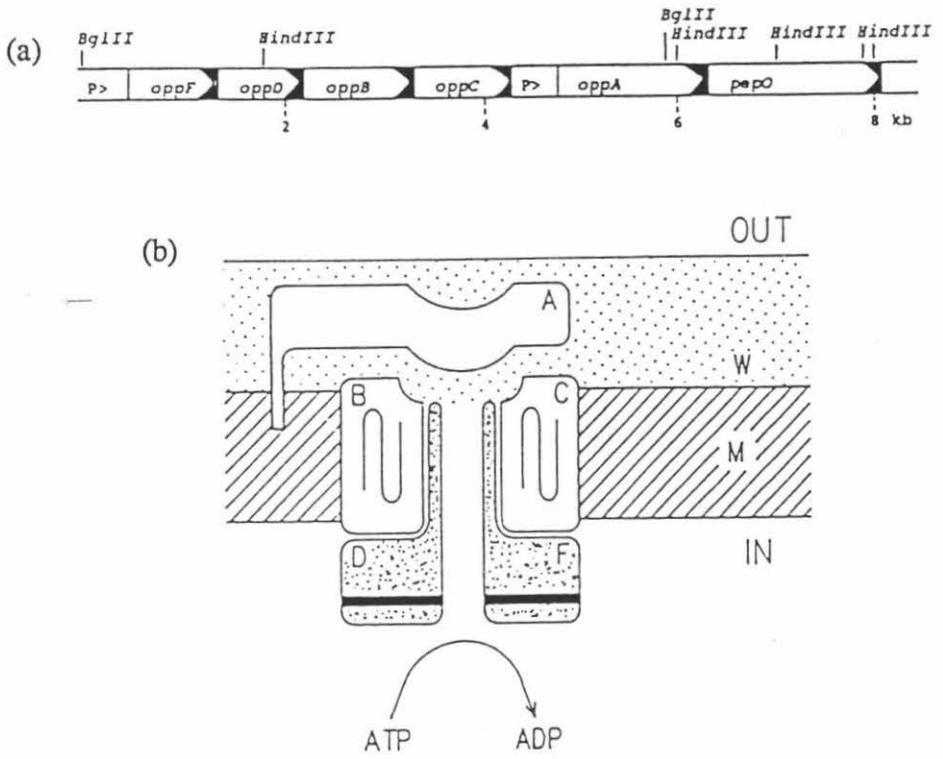


Figure 1.2.3.2

(a) Genetic organisation of the *opp* operon of *L. lactis*

(b) Proposed model of the *L. lactis* oligopeptide system encoded for by the *opp* operon (Kok and de Vos, 1994).

The inability of other mutants of strain ML_3, in which either *opp A* or the entire operon were deleted, to grow in milk demonstrated that this transport system was essential to casein utilisation (Tynkkynen *et al.*, 1993; Yu , 1994).

Initial findings also suggested that the activity of this transport system was dependent upon the nature of the nitrogen source present in the medium (Kunji *et al.*, 1993).

1.3 Peptide metabolism and its significance.

Peptide metabolism can be viewed as those processes involved in the transformation of casein-derived oligopeptides into an intracellular pool of metabolisable amino acids. This is a significant area of research for two principle reasons.

(a) Lactococcal nutrition.

Although proteinase activity is essential to the degradation of milk proteins, further metabolism of the oligopeptides to free amino acids is essential to supply the amino acid requirements for growth because of the low amount of free amino acids available in milk.

Supplying milk-grown, proteinase positive and proteinase negative strains with small peptides and free amino acids improves both growth rates and yields (Thomas and Mills, 1981).The maximum growth rates attained when lactococci are grown in media containing peptides as sources of essential amino acids are higher than those in media containing amino acids alone (Hugenholtz *et al.*, 1987).

A comprehensive understanding of peptide metabolism is therefore essential for success in the pursuit of faster growing strains for industry.

(b) Product quality.

Peptide metabolism influences the flavour and texture of cheeses. Distinct flavour characteristics such as bitterness are a consequence of the accumulation of peptides not utilised by starter bacteria during both the initial period of cell growth and while the cheese is ripening (Stadhouders *et al.*, 1983; Visser *et al.*, 1983).

Certain strains characteristically produce savoury cheeses while others typically produce bitter ones. This implies important differences in the mechanisms by which these strains metabolise milk peptides. An understanding of these differences should enable us to manipulate starter cultures to produce cheeses with consistently favourable organoleptic properties.

1.4 The role of peptide metabolism in the lactococcal proteolytic pathway.

While peptide metabolism encompasses the action of both peptidases and transport systems, the relative contribution of each to the utilisation of casein peptides is much debated.

1.4.1 Current models of peptide metabolism

Two models can be proposed from the research available to date.

(a) Extracellular cleavage precedes transport.

In this model extracellular peptidases act to degrade the oligopeptides produced by the cell wall-associated proteinase into smaller peptides, principally di-/tripeptides, and free amino acids (see Figure 1.4.1.1).

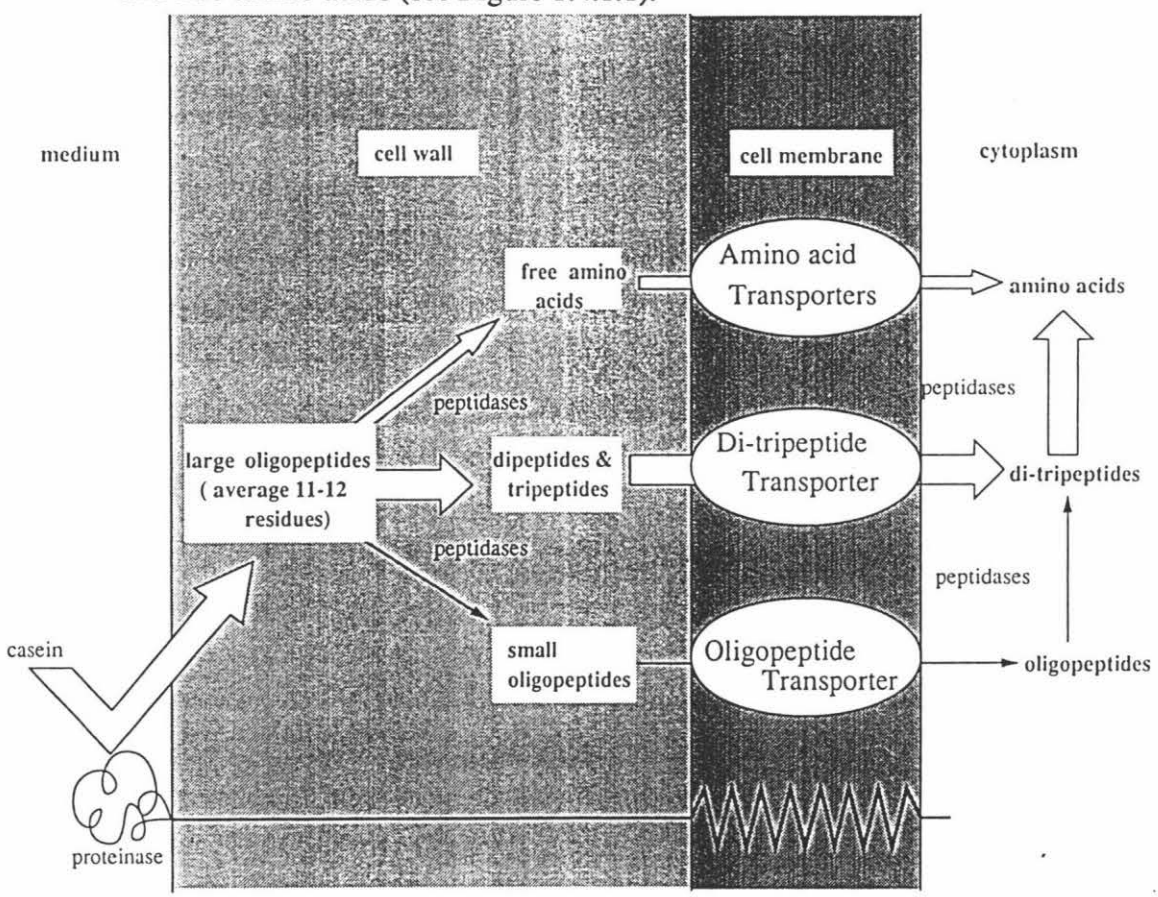


Figure 1.4.1.1: A possible organisation of the lactococcal proteolytic pathway. Current evidence indicating low relative rates of activity of the oligopeptide transport system (Smid, 1991; Kunji *et al.*, 1993), support this model which favours a predominant role for the di-/tripeptide transport system

It has been predicted from knowledge of the type of oligopeptides produced by the cell wall-associated proteinases, that the extracellular peptidase complement would have to involve at least a general aminopeptidase, an X-prolyl dipeptidyl aminopeptidase, and a glutamyl aminopeptidase (Smid, 1991).

Other studies have implicated an extracellular presence for a dipeptidase (Kolstad and Law, 1985), a tripeptidase (Bacon *et al.*, 1993; Sahlstrom *et al.*, 1993), a general aminopeptidase (Exterkate, 1984), an aminopeptidase A (Exterkate and de Veer 1987a), and an endopeptidase (Yan *et al.*, 1987a; O'Harte *et al.*, 1993).

However, the results of many of these studies are not conclusive, a major cause of uncertainty lying in the difficulty of obtaining cell fractions free from intracellular enzyme contamination. For example, the "cell wall" associated dipeptidase identified by Kolstad and Law (1985) was found to have specificity profiles very similar to that of its intracellular counterpart.

While it is possible that some extracellular peptidase activity could result from cellular leakage itself, this is unlikely to become significant until the later stages of growth when cell numbers are already at high densities.

The recent isolation and sequencing of genes encoding lactococcal peptidases has provided evidence against an extracellular location for peptidase activity, as none of the peptidases characterised to date has the leader sequence characteristic of extracellular targeted proteins (Kok and de Vos, 1994).

(b) Transport precedes intracellular cleavage.

In this model the products of proteinase-catalysed casein degradation are transported directly into the cell after which they are rapidly degraded by internal peptidases (see Figure 1.4.1.2).

However, the demonstration that the di-/tripeptide carrier system is essential for growth of the *cremoris* strain ML_3, necessitates a more extensive cleavage of β–casein than is currently known to be the case (Smid *et al.*, 1989b).

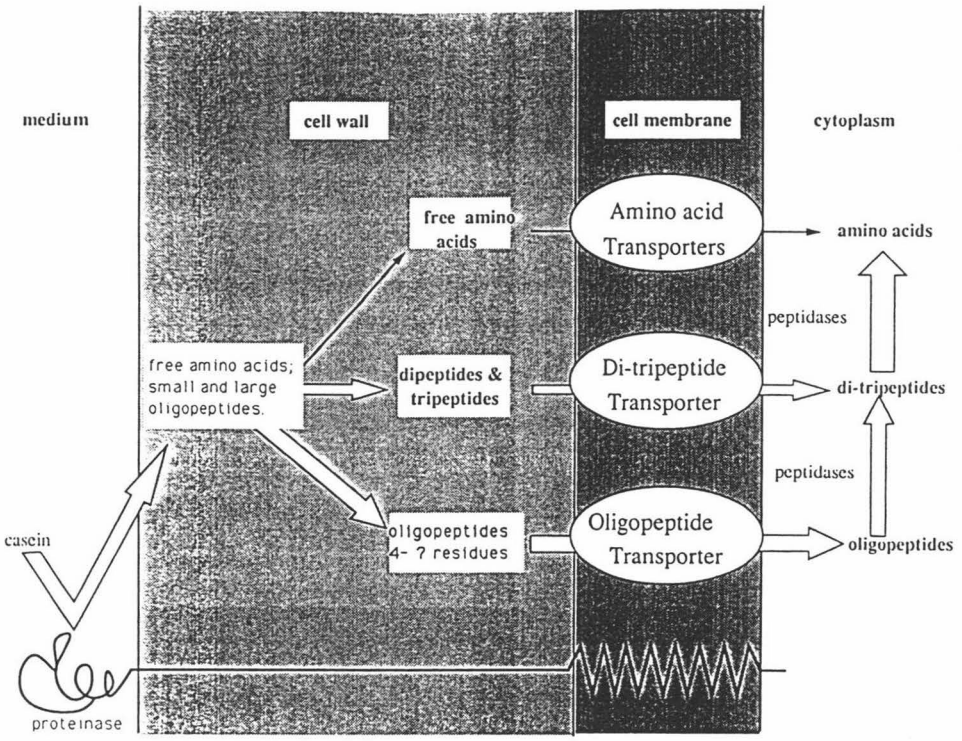


Figure 1.4.1.2. An alternative organisation of the lactococcal proteolytic pathway.

Current evidence on the identity of the peptide products of casein hydrolysis by the cell wall-associated proteinase (Monnet *et al.*, 1989; Visser *et al.*, 1988, 1991, 1994; Reid *et al.*, 1991a, 1991b, 1994) indicate that the minimum size of these peptides is 6 to 7 amino acid residues. However, it is possible that smaller products are not being detected by the analytical techniques used. Alternatively, small peptides may not be produced by the proteinase when it is released from the cell wall for *in vitro* studies.
If all the peptidases are eventually shown to have an intracellular location, then the extent to which lactococcal proteinases can degrade casein will be an area requiring extensive research.

This model implies a predominant role for the oligopeptide transport system. Current knowledge (Smid, 1991; Kunji *et al.*, 1993) does not suggest that this system is capable of transporting a sufficient range of larger peptides at a rate which would support rapid growth. However these conclusions come from a small number of studies involving only a single lactococcal strain. Furthermore the results of these are in conflict with other earlier research done in this area as discussed previously.

More information is therefore needed about this transport system particularly regarding the upper size limit of peptides it will transport, substrate specificity and the relative rates of peptide transport.

1.4.2 Strain Differences in Peptide Metabolism.

Most of the work carried out on peptide transport systems has come from the studies in Koning's laboratory in Groningen on the *cremoris* strain ML_3 and its mutants. While these have provided us with valuable information, they have not questioned whether the ML_3 strain is "typical" of the major cheese producing strains.

Law (1977) found that there were actually three quite distinct groups with regard to peptide utilisation.

(1) Those *lactococci* that transport dipeptides prior to hydrolysis ie the dipeptides are transported as such.

(2) Those *lactococci* that transport the amino acid constituents after dipeptide hydrolysis

(3) Those *lactococci* that did not transport dipeptides at all.

This raises important questions about the validity of applying the knowledge obtained from the ML_3 strain to the *lactococci* as a group.

1.5 Aims of the Current Study

The central objective of this research is to investigate aspects of peptide utilisation by whole lactococcal cells. The results from this work will hopefully provide answers to some important questions in this area.

(1) Is the oligopeptide transport system capable of transporting large peptides and at physiologically relevant rates ?

(2) Are there any significant differences in the process of peptide utilisation between the ML_3 strain and other strains ?

(3) What peptidases are essential for growth ?

(4) What factors regulate the enzyme component of peptide utilisation?

An attempt to answer some of these questions will involve the following experimental strategies:

(a) The synthesis of a homologous series of peptides of increasing size from the dipeptide Val-Gly to the decapeptide Val-Gly_9. Valine was chosen as the amino acid on which to base this series as is essential to the growth of both *lactis* and *cremoris* strains (Reiter and Oram, 1962).

(b) Peptide uptake studies with this series of peptides by the *cremoris* strains ML_3, E_8, and SK_{11}, and the *lactis* strains 1403 and 920.

(c) Growth studies with these same strains in chemically defined media, where free valine is substituted for by the Val-$(Gly)_x$ series of peptides.

(d) Peptide uptake studies using oligopeptides isolated by HPLC from a proteinase digest of β-casein.

(e) The isolation and characterisation of mutants deficient in peptide utilisation from one lactococcal strain.

(f) The investigation of the effect of nitrogen source type upon the expression and activity of intracellular peptidases.

Chapter Two: Materials and Methods.

2.1 Materials

2.1.1 Bacterial strains

The following lactococcal strains were obtained from the New Zealand Dairy Research Institute (NZDRI) Palmerston North.

Lactococcus lactis subsp *lactis* 1403
Lactococcus lactis subsp *lactis* 920
Lactococcus lactis subsp *cremoris* E_8
Lactococcus lactis subsp *cremoris* ML_3
Lactococcus lactis subsp *cremoris* SK_{11}
Lactococcus lactis subsp *cremoris* AM_2
Lactococcus lactis subsp *cremoris* AM_1

Lactococcus lactis subsp. *lactis* KG301 was supplied by Dr L. McKay, University of Minnesota.

2.1.2 Growth media constituents

Low heat reconstituted skim milk powder was obtained from NZDRI; biotrypticase from bioMerieux, France; yeast extract from Gibco laboratories UK; beef extract from BBL Microbiological Systems USA, and D (+) lactose from Reidel-de-Haen, Germany.
Free L (+) amino acids were obtained from either Sigma or BDH; nitrogen bases from BDH; vitamins and minerals from Sigma; β-casein from Sigma, and bacteriological agar from Scientific Supplies, NZ.

2.1.3 Substrates for enzymes

All peptides were obtained from Sigma unless otherwise specified; L-lysine-nitroanilide (lys-*p*Na) was supplied by Bachem Inc.; L-glycylproline-7-amido-4-methylcoumarin (Gly-Pro-AMC) from Mr Richard Lloyd, Massey University; and fluorescein isothiocyanate-conjugated β-casein (FITC β-casein) from Mr Julian Reid, Massey University.

2.1.4 Peptide synthesis materials

tert-butylcarbonyl valine (Boc-Val) and *tert*-butylcarbonyl alanine (Boc-Ala) were supplied by Bachem, California USA; β-chloroalanine, N-hydroxysuccinimide, dichlorocarbodimide, tri-L-glycine and di-isopropylethylamine from Sigma; dioxan from Ajax, Australia; 1,1,3,3 tetramethylguanidine from Eastman Kodak, USA; acetonitrile from Mallinckrodt, USA; and propan-2-ol from May and Baker Ltd, England.

2.1.5 Peptide uptake materials

Val-Gly, and Val-Gly_2 were obtained from Sigma; Val-Gly_3, Val-Gly_4, Val-Gly_7 and Val-Gly_9 were either synthesised by the author or supplied by the Massey University Separation Science Unit as indicated; sodium orthovanadate and carbonyl cyanide tri-chlorophenyl hydrazone (CCCP) from Sigma; TFA from Habcarbon, USA; D (+) dextrose from United states Biochemical Corporation; and 20 mM sodium citrate solution pH 2.5 from Applied Biosystems.

2.1.6 Miscellaneous materials

Fructose 1,6 bisphosphate, triosephosphate isomerase and α-glycerophosphate dehydrogenase from Sigma; bicinchoninic acid from Sigma; and bovine serum albumin (BSA) from Sigma.
All other reagents and solvents were of analytical grade wherever possible.

2.2 Methods

2.2.1 Growth media

(a) Skim milk medium (SMM).

A weighed amount of reconstituted skim milk powder was mixed into a paste with a small amount of hot water. When homogeneous this was made up to a final concentration of 10 percent (w/v) with distilled water. It was then sterilised by autoclaving at 10 psi for 10 minutes (min).

(b) Undefined lactose broth medium (UBM).

Components per litre of medium;

Lactose 20 g	Potassium dihydrogen phosphate (KH_2PO_4) 5g
Peptone 10 g	Magnesium sulphate 0.2 g
Yeast extract 10 g	Manganese chloride 0.05 g
Beef extract 2 g	distilled water to 1 L

Before making up to volume the pH was adjusted to 7.0 with 10 M NaOH. The medium was sterilised by autoclaving at 15 psi for 15 min.

(c) Chemically defined medium (CDM).

A chemically defined medium based on a comparison of defined media used by other workers for the growth of lactic acid bacteria was used for much of the work described in this study. A comparison of the amino acid composition of the medium described below with these media is presented in Table 2.2.1.1.

Table 2.2.1.1 Comparison of the amino acid composition of chemically defined media used by researchers for the growth of lactococci.

amino acid.	Concentration ($mg.l^{-1}$) in the growth media designed by the following research groups.						
	Present study 1994	Ford 1962	Reiter 1962	*Crow	Poolman 1987	Jenson 1993 (i)	Jenson 1993 (ii)
ala	**600**	770	300	300	240	303	
asp	**600**	770	600	300	420		
asn	**200**			100	350	106	106
cys	**200**	308		100		97	
gln	**200**			300	390	102	102
gly	**200**	308	200	100	175	203	
lys	**800**	770	800	200	440	205	
phe	**200**	308	200	100	275	198	
pro	**200**	308	540	100	675	299	
ser	**200**	308	400	200	340	305	
thr	**200**	308	200	100	225	202	
trp	**200**	308	200	100	50	102	
tyr	**200**	308	100	100	200	54	
arg	**200**	308	450	100	125	191	
glu	**600**	1538	3200	300	500	309	3089
his	**400**	308	550	100	150	47	47
ile	**400**	770	200	100	210	105	105
leu	**400**	770	200	100	475	105	197
met	**400**	308	140	100	125	105	75
val	**400**	770	300	100	325	105	305

* Personal communication.

This medium was constructed from 5 separate components

(i) Nitrogen component (amino acids and nitrogen bases)

A	600 mg	F	200 mg	E	400 mg
D	600 mg	P	200 mg	L	400 mg
N	200 mg	S	200 mg	I	400 mg
C	200 mg	T	200 mg	H	400 mg
G	200 mg	Y	200 mg	M	400 mg
Q	200 mg	R	200 mg	V*	400 mg
W	200 mg	K	800 mg		

adenine	10 mg
uracil	10 mg
xanthine	10 mg

These constituents were dissolved in 400 ml of distilled water by boiling for 10 min.

* Note: Valine could be omitted from the medium at this stage. This allowed for its substitution later by an equimolar amount of one of the following valine-containing peptides: Val-Gly; Val-Gly_2; Val-Gly_3; Val-Gly_4; or Val-Gly_7

(ii) Guanine component

A stock solution was prepared by dissolving 50 mg of guanine solid in 25 ml 1N HCl. This was stored at -20 °C until required.

(iii) Buffer component

Sodium acetate	1.0 g
EDTA	4 mg
Disodium hydrogen phosphate (Na_2HPO_4)	8.5 g
KH_2PO_4	2.0 g

These constituents were dissolved and made up to 550 ml with distilled water

(iv) Carbohydrate component

Lactose 15 g
Magnesium sulphate 2.5 g

These were dissolved into 50 ml dH_2O by heating to 100 °C, and then sterilised by autoclaving at 15 psi for 15 min..
Note: This component was stored as 10 ml batches because the lactose would often precipitate out of solution within 4-5 days of being opened.

(v) Vitamin - mineral component

PABA	20 mg	Pyridoxine	100 mg
Biotin	10 mg	Thiamine	10 mg
Folic acid	10 mg	$FeSO_4.2H_2O$	100 mg
Nicotinic acid	100 mg	$MnCl_2.4H_2O$	100 mg
Pantothenic acid	100 mg	Tween 80	10g or 10 ml
Pyridoxal HCl	20 mg	Riboflavin	5 mg

These constituents were dissolved into 90 ml distilled water (this process can be facilitated by warming in hot water. The riboflavin is very difficult to dissolve).The resulting solution was then filter-sterilised through a 0.22 µm Millipore filter into presterilised 20 ml screw top bottles (stbs).This was stored as 10 ml batches at -20 °C until required for use.
Note: It was found that if left at room temperature for any length of time, the solution oxidised to a dark brown colour.

To prepare the chemically defined medium, the first three components were mixed together in the following amounts; 400 ml (i) + 0.5 ml (ii) + 550 ml (iii). This 950.5 ml of stock medium was buffered to pH 7.0 and then sterilised by autoclaving at 15 psi for 15 min. Components (iv) and (v) were aseptically added to the stock medium of components (i)-(iii) just prior to inoculation with cells. To every 95 ml of stock medium, 5 ml of component (iv) and 300 µl of component (v) were added.

(d) Casein hydrolysate medium (CHM)

Components per litre of medium.

Pancreatic digest of casein	5 g	Adenine	10 mg
Na acetate	1 g	Uracil	10 mg
EDTA	4 mg	Xanthine	10 mg
Na_2HPO_4	8.5 g	Guanine	500 µl of a 2 mg ml^{-1} soln
KH_2PO_4	2.0 g	distilled water to 1 L	

After mixing, the components were buffered to pH 7.0 with 10 M NaOH and then sterilised by autoclaving at 15 psi for 15 min.
To complete the medium, lactose and vitamin-mineral solution were added at concentrations described previously for the CDM in the previous section .

(e) 1 % β-casein defined medium (β-CDM).

Components per 100 ml of medium.

Sodium acetate	2.1 mg	Adenine	1.05 mg
2 glycerophosphate	2.15 g	Uracil	1.05 mg
KH_2PO_4	136 mg	Xanthine	1.05 mg
EDTA	0.4 mg	Guanine	52 ml of a 2mg.ml^{-1}soln
L-histidine	42 mg		
distilled water	95 ml		

These components were dissolved by heating to 100 °C for 5 min. When the medium had cooled to room temperature, 2 ml of an 8.83 mg. ml^{-1} calcium chloride solution were added (final concentration 2 mM). β-casein (1g) was then dissolved gradually over 10 min.
The medium was sterilised by passing through a 0.22 µM Millipore filter into a presterilised Shott bottle in which it was stored at 4 °C until required.

For growth experiments, 10 ml aliquots were aseptically transferred into presterilised stbs. Lactose and vitamin-mineral solution were then added to concentrations described previously for CDM in Section 2.2.1c.

2.2.2 Preparation of agar plates

Solid versions of the different media described in Section 2.2.1 were prepared by addition of bacterial agar into the various media to a final concentration of 1.5 g per 100 ml before sterilising at 15 psi for 15 min.

2.2.3 Maintenance of bacterial cultures

Cultures of lactococci were grown in SMM to the point of coagulation. For long term storage these milk cultures were frozen at -70° C. For short term storage, milk cultures were maintained at 4 °C, for a maximum of 14 days, after which fresh cultures were grown up from the -70 °C stocks.

2.2.4 Growth studies

The growth of lactococcal strains in various defined media was studied as follows

(i) As small scale cultures in 20 ml stbs

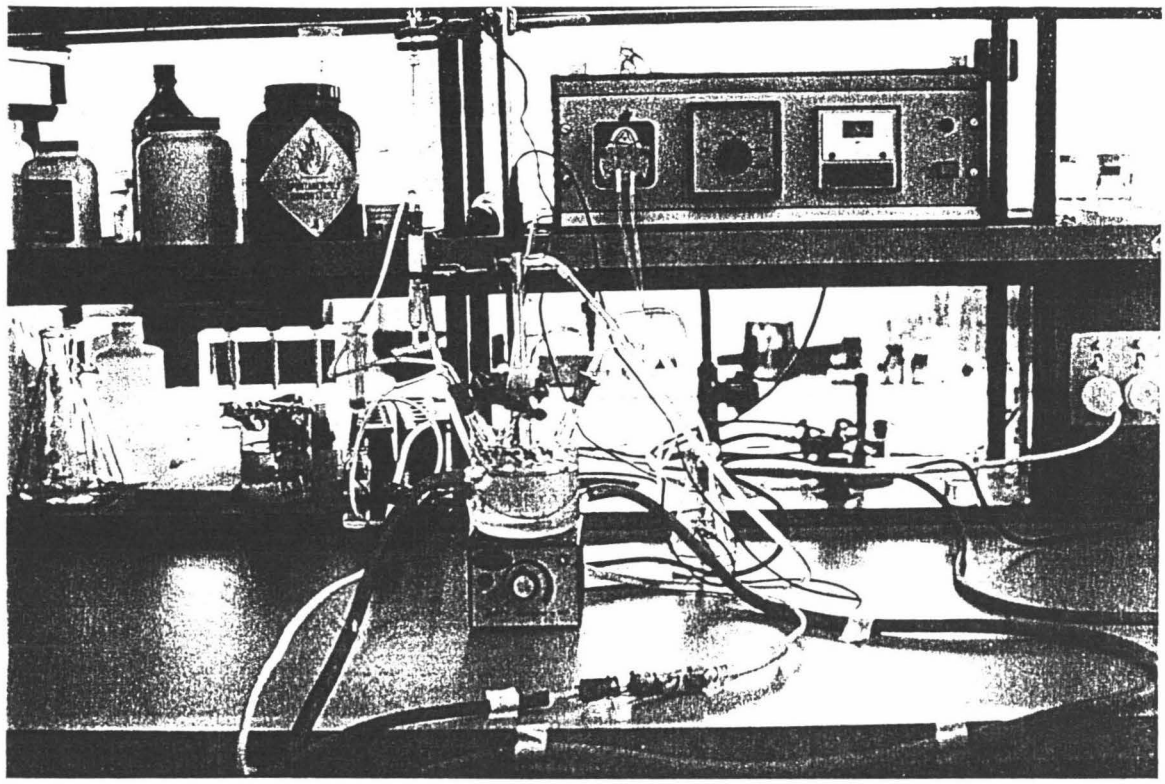
These were used when there was no need to harvest the cells for enzyme assays at the end of growth, or when a peptide of interest was available only in amounts that permitted growth studies on a miniature scale. Media were usually buffered only by their initial buffer components. The major exception to this was the β-casein defined medium to which sterilised 0.5 M NaOH was added during growth of cultures to keep the β-casein from precipitating due to the pH decrease.

Once the media were inoculated, the bottles were incubated at 30 °C for the duration of the experiment. Samples for assay were taken by aseptically withdrawing small volumes.

(ii) As large scale cultures in a 200 ml fermenter flask

This was used if there was an intention to harvest the cells for assay of peptidase activities. The medium was maintained at 30 °C by sitting the flask in a thermostatted water bath. The cells were gassed with 5 percent CO_2 in nitrogen,

and the pH was maintained at 6.0 - 6.3 by automatic alkali addition. Samples were withdrawn aseptically into collection bottles by a syringe induced vacuum (Figure 2.2.4.1).



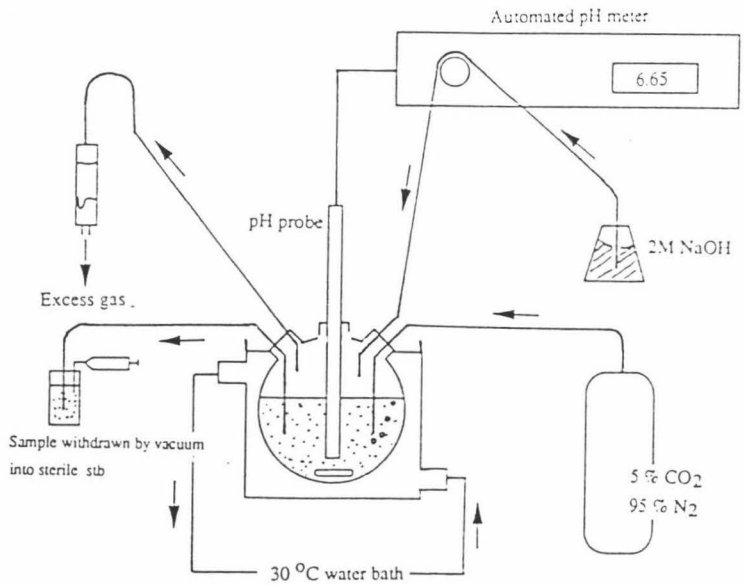


Figure 2.2.4.1 A photograph and diagrammatic representation of the system used to grow lactococci in a 200 ml fermenter flask.

In both cases the protocol used for preparing the inoculum was the same.
In preparing the inoculum for cultures to be grown in defined media, milk-grown cells were passed through one generation of UBM and two generations of the medium of interest. These passages are necessary to prevent carry-over of nutrients from more complex media to an extent that could influence the results. By determining the extent of growth in CDM lacking the essential amino acid valine, it was shown that carry over was detectable until the third passage (Figure 2.2.4.2). Growth was measured by following the increase in turbidity of a sample at 540 nm, and by the decrease in the pH of the medium. At the end of the experiment, samples were plated onto UBM agar plates to check for any yeast or bacterial contaminants. Purity was also monitored during growth by use of the Gram stain.

2.2.5 Harvesting of cells for peptidase determinations

Cells were harvested from the fermenter as they were entering the stationary phase of growth.

(i) Cells grown in media other than milk

The cells were separated from the medium by centrifuging at 8000 g for 10 min at 4 °C. The cell pellet was resuspended in 0.05 M sodium acetate-phosphate ($NaOAc/PO_4$) pH 6.4 and then recentrifuged using the same conditions. This washing procedure was repeated, the pellet weighed and then stored at -20 °C until required.

(ii) Cells grown in milk (SMM)

To collect the bacteria, the SMM was initially buffered to a pH of 7 with 1 M NaOH. The milk culture was then placed on ice until its temperature had dropped below 10 °C. A 6 ml aliquot of 25 percent sodium citrate was added for every 100 ml of medium. This was left for 15 min on ice to disperse any protein micelles left in the milk culture. The cells were recovered by centrifuging at 8000 g for 10 min at 4 °C. The recovered pellet was washed twice in 0.05 M $NaOAc/PO_4$ pH 6.4 as described above.

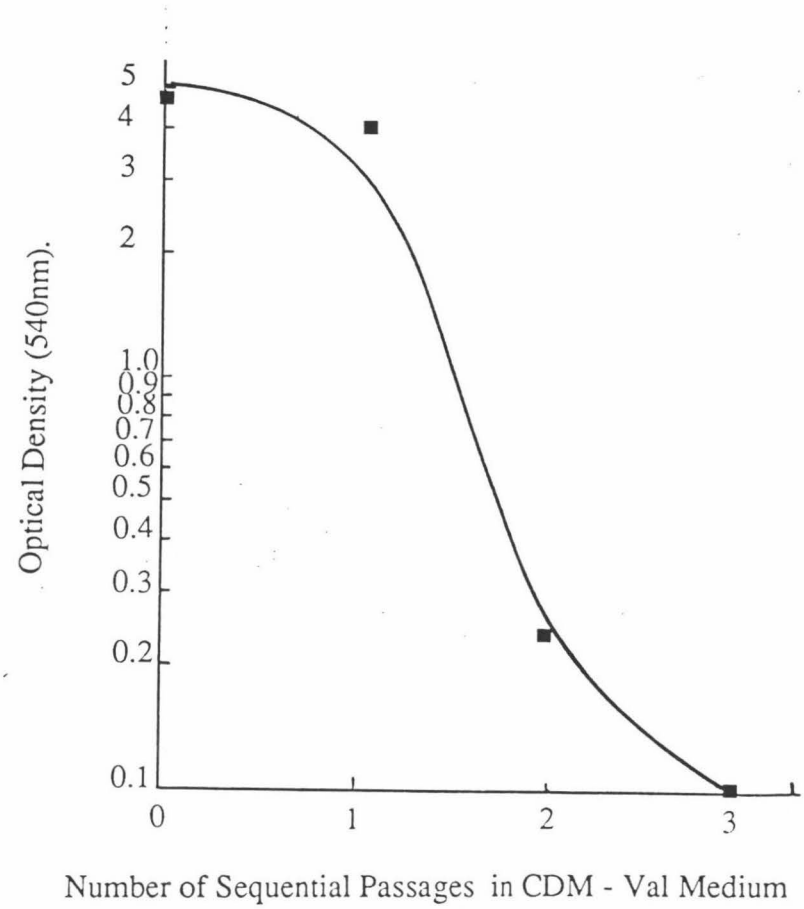


Figure 2.2.4.2 Demonstration of the necessity for two successive passages when preparing an inoculum for culture growth on chemically defined medium.
To determine the minimum number of passages required to prevent nutrient carry-over when transferring cells from one medium type to another, *L. lactis* subsp *lactis* 1403 was grown through three sequential transfers of chemically defined medium lacking the essential amino acid valine. An aliquot (1 ml) of broth-grown cells (generation 0) was used to inoculate the first passage in CDM - Val. After 8 h growth, a 1 ml aliquot of this culture were used to inoculate a new passage of CDM - Val. This procedure was repeated for a maximum of three transfers. The ordinate scale shows the optical density (540nm) attained after incubation for 8 h.

2.2.6 Peptidase assays

After thawing, the frozen cell pellet (between 200-500 mg wet weight) was resuspended in 5-10 ml of ice-cold 20 mM phosphate buffer , pH 6.4. The cells were disrupted by passage through a cooled French pressure cell at a pressure of 38 MPa. The cell debris was removed by centrifugation at 8000 g for 10 min at 4 °C, and the cell-free supernatant kept on ice.

Assays for six different peptidases were carried out:

(i) dipeptidase, tripeptidase, prolidase and proline/iminopeptidase

The assay procedure for these peptidases is based on the fluorescence of amino acids after reaction with *o*-phthaldialdhyde in the presence of 2-mercaptoethanol (Roth, 1971)

The fluorescence yield of free amino acids is much stronger than that of peptides. Enzymatic hydrolysis of a peptide can therefore be followed as an increase in the overall fluorescence of the reaction mixture (Taylor and Tappel,1973).

All four peptidase activities were assayed for by the following protocol

Reaction mixture	
20 mM phosphate buffer pH 6.8	800-950 µl
Substrate (5 mM or 2 mM - see below)	500 µl
Enzyme extract (may need to be diluted 5-10 fold)	50-200 µl

Fluorometric assay reagent	
0.05 M borate buffer pH 9.5	9.5 ml
2-mercaptoethanol (5 mg. ml $^{-1}$ in EtOH)	1.5 ml
o-phthaldialdhyde (10 mg. ml^{-1} in EtOH)	1.5 ml

The reaction mixture was incubated at 30 °C. After addition of the enzyme fraction to the reaction mixture, 200 µl samples were removed at timed intervals and mixed immediately with 100 µl of 0.1 M acetic acid.

When all the necessary samples were taken, 2.7 ml of freshly made assay reagent were added to each. The fluorescence was measured immediately on an AMINCO SP500 Ratio Spectrofluorometer (excitation λ = 340 nm; emission λ = 455 nm).

The substrates used for the different enzymes were as follows:

Dipeptidase	5 mM Ala-Ala
Tripeptidase	5 mM Leu-Gly-Gly
Prolidase	5 mM Leu-Pro
Proiminopeptidase	2 mM Pro-Ala

(ii) lysine aminopeptidase

Enzyme activity was assayed for using the chromogenic substrate L-lysine *p*-nitroanilide (lys-*p*NA). When released by hydrolysis, the free *p*-nitroaniline produces a yellow colour which can be followed spectrophotometrically at 405 nm.

The following protocol was used:

100 mM MES buffer pH 6.8	800 µl
5 mM lys-pNA	100 µl
enzyme extract	100 µl

The increase in absorbance at 405 nm was measured at room temperature in a 1 ml quartz curvette using a Hitachi U1101 spectrophotometer .

(iii) X-prolyl dipeptidyl aminopeptidase.

Enzyme activity was assayed using the substrate L-glycyl-proline-7-amido-4-methylcoumarin (Gly-Pro-AMC). Cleavage of the amino acyl bond results in liberation of AMC, which unlike the parent compound, has a strong fluorescence (excitation λ =385 nm; emission λ = 460 nm).

The following protocol was used:

100 mM MES buffer pH 6.8	1.30-1.75 ml
1 mM Gly-Pro-AMC	200 µl
enzyme extract	50-500 µl

The reaction mixture was incubated at room temperature. An increase in the relative fluorescence ratio was followed in an AMINCO SP500 Ratio Spectrofluorometer, which had been standardised using a 100 µM solution prepared from recrystallised AMC.

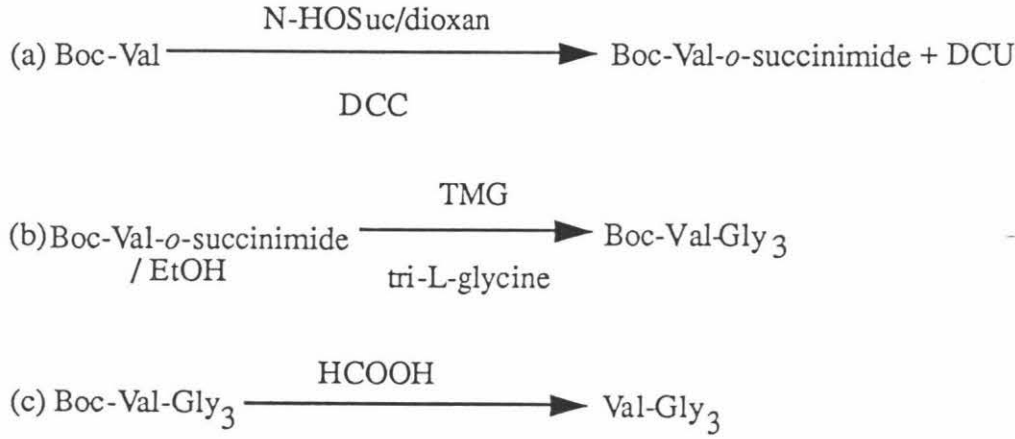
2.2.7 Protein determinations

Protein concentration was determined using the bicinchoninic acid method (Smith *et al.*, 1985). A reaction mixture was prepared by adding 1 ml of copper sulphate pentahydrate 4 % solution to 50 ml of bichinchoninc acid solution. An aliquot (2 ml) of this mixture was added to 100 µl of the sample and incubated at 37 °C for 30 min. After cooling for 5 min, the absorbance of each sample was read at 562 nm.
The gross amount of protein present in each sample was quantified by comparison to a standard curve made from bovine serum albumin, covering the range 0-50 µg protein. Duplicates of both standards and unknowns were routinely used.

2.2.8 Synthesis of Val-Gly$_X$ peptides

(i) Synthesis of Val-Gly$_3$.

This peptide was prepared using a solution-phase activated ester method (Anderson et al., 1964). The chemistry involved is summarised as follows:



A weighed sample (0.01 mol) of tertiary-butyloxycarbonyl-valine (Boc-Val) was added to a slight molar excess of N-hydroxysuccinimide (N-HOSuc). 15 ml of dioxan was added and the reagents mixed over iced water for 15 min. 0.01 mol of dicyclohexylcarbodimide (DCC) were added and the mixture stirred at room temperature for 3 h.
At the end of this time the dicyclohexylurea (DCU) was filtered off by vacuum filtration and the remaining solution taken to dryness using rotary evaporation.
The residue was redissolved in 10-15 ml of 95 % EtOH. Tri-L-glycine (0.02 mol) was separately dissolved into 0.02 mol of 1,1,3,3-tetramethylguanidine (TMG) over a half hour period. This solution was then added to the EtOH mixture

After overnight stirring at room temperature, the EtOH was evaporated off to leave a viscous oil. This was treated with 10-15 ml of 10 % H_2SO_4 and solid NaCl was added to ensure complete insolubility of the Boc-Val-Gly$_3$ in the aqueous layer. The mixture was then shaken with ethyl acetate in a separating funnel. This washing was repeated 2-3 times to extract all the crude peptide into the organic layer. The pooled ethyl acetate washings were then dried down by rotary evaporation.

The Boc-peptide was deprotected by stirring with 100 % formic acid for 2-3 h at a ratio of 80 ml of acid for every 2.8 g of crude product (Halpern *et al.*, 1967). The formic acid was then evaporated off and the product redissolved in 20 ml of glacial acetic acid. This was freeze dried down to give approximately 1.5 g of a crude viscous oil.

At this point the following checks were made to confirm the identity of the crude peptide:

(i) a sample of the oil was dissolved up in water and subjected to separation by High Performance Liquid Chromatography (HPLC), on a Philips PU4100 HPLC with a Vydac 218TP, C18, 4.6 mm by 250 mm column (pore size = 10 μm; pore diameter = 300 Å). The absorbance of the eluate was monitored at 220 nm on a PU4110 UV/VIS detector. Solvent A was 0.1 % TFA in deionised-distilled water. Solvent B was 0.08 % TFA in acetonitrile. The one major peak that eluted at 5.4 - 5.8 % solvent B was collected from repeated runs.

(ii) pooled samples of the major peak were subject to hydrolysis in 6 M HCl under vacuum for 24 h at 110 °C. The product of this digestion was analysed using an automated Pharmacia LKB 4151 amino acid analyser.

(iii) further samples were dried down and subject to molecular weight determination by liquid secondary ion mass spectrometry (SIMS).

Having confirmed the identity of the crude peptide to be Val-Gly_3, it was purified from contaminants by repeated isocratic HPLC runs in 100 % A on a Synchroprep preparative C18, 10 x 250 mm, column (silica # R503; pore diameter = 30 μm; flow rate = 2 ml min^{-1}).
A 15 mg ml^{-1} solution of the crude peptide was prepared. Aliquots (3 ml) of this were loaded per HPLC run. Analysis of fractions from the initial run showed that the Val-Gly_3 eluted very early. The following step gradient profile was therefore used in the cleaning up of the crude peptide.

	Injection
gradient 1:	100 % solvent A for 15 min
gradient 2:	100 % solvent A to 100 % solvent B in 10 min
gradient 3:	100 % solvent B for 10 min

gradient 4:	100 % solvent B to 100 % solvent A in 10 min
gradient 5:	100 % solvent A for 20 min (to allow the column to re-equilibrate).
	Next injection

The pooled fractions from these separations were evaporated down to give a crystalline solid. A yield of 150 mg of the purified peptide was obtained from 400 mg of the crude peptide.

(ii) Synthesis of Val-Gly_4

Several attempts were made to synthesise this peptide by the activated ester method employed for Val-Gly_3. However, analysis of the ethyl acetate extracts by HPLC failed to show any major peaks. Three peaks were found when the aqueous washings were analysed, but none had a valine to glycine ratio of 1:4 when subject to amino acid analysis.

Thereafter solution phase synthesis was abandoned in favour of solid phase peptide synthesis (SPPS). Val-Gly_4 was synthesised by the Massey University Separation Science Unit (SSU), using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry on an Applied Biosystems 430A automated peptide synthesiser. The peptide was supplied in a crude state.
After the identity of the major peak was confirmed to be Val-Gly_4 by amino acid analysis and molecular weight determination, it was purified from contaminants using preparative HPLC as described above for Val-Gly_3. Freeze drying of the purified fractions yielded 120 mg of an oil residue. This was stored at -20 °C until required.

(iii) Preparation of Val-Gly_7

Val-Gly_7 was synthesised by Massey University SSU using Fmoc based SPPS.
45 mg of purified peptide were supplied. The identity of this peptide was confirmed by amino acid analysis and molecular weight determination. It was stored at -20 °C until required.

(iv) Preparation of Val-Gly9

Val-Gly9 was synthesised by Massey University SSU using Fmoc based SPPS. 14 mg of purified peptide were supplied. The peptide was stored at -20 °C until required.

2.2.9 Isolation of β-casein peptides.

(i) Preparation of a crude cell-wall associated proteinase extracted from *L. cremoris* E8

A culture of *L. cremoris* E8 culture was grown in 900 ml of sterilised SMM to which had been added 27 ml of sterilised 2.5 M sodium glycerophosphate buffer (to prevent coagulation of the milk by the lactic acid produced during bacterial growth). The cells were grown overnight at 30 °C until a final pH of about 5.3 was achieved.

The cells were separated from the remaining milk constituents as described previously in Section 2.2.4. They were then suspended in 25 ml cold 50 mM PO_4 pH 6.4 . The cells were incubated at 30 °C for 30 min to allow for the cell wall-associated proteinase to be released from the cells (Thomas and Mills, 1981). Incubation of lactococcal cells in Ca^{2+}-free buffer is known to result in the release of most of the cell-wall bound proteinase by a process of autoproteolytic cleavage at a site close to the C-terminal end of the enzyme (Laan and Konings, 1989).
The proteinase-containing supernatant was recovered by centrifuging the suspension at 8000 g for 10 min at 4 °C. The resuspended cell pellet was incubated for a further 30 min and then centrifuged again . The supernatants were then combined and centrifuged to remove any particulate matter. The solution decanted from this step was concentrated to about 5 ml in an Amicon ultrafiltration system using an XM50 membrane at 4 °C. The resulting crude proteinase was stored on ice until required.

(ii) Assessment of proteinase activity

The activity of the crude proteinase was determined using the method of Twining (1984). This method is based on the measurement of TCA-soluble fluorescent peptides released by the enzymatic cleavage of fluorescent isothiocyanate-conjugated β-casein (FITC β-casein).

Eppendorf tubes were set up covering a range of crude enzyme volumes as follows:

crude extract (µl)	0.5M NaOAc/PO4 pH 6.4 (µl)	FITC β-casein (µl)
10	190	100
20	180	100
40	160	100

The reaction mixture was incubated at 25 °C. Immediately after the addition of the FITC β-casein the eppendorf was shaken thoroughly and two 60 µl samples were each added to 150 µl of 5 % (w/v) TCA. This step was repeated after 60 min. After 20 min the yellow precipitate of undigested FITC-β-casein was removed by spinning the samples on a bench centrifuge at 12000 g for 5 min.
An aliquot (100 µl) of the supernatant was removed, care being taken to ensure that no precipitate was also removed. This was added to 3 ml of 0.5M Tris/HCl buffer pH 8.5.

The relative fluorescence (RF) ratio of these samples was measured on an Aminco SP500 Ratio Spectrofluorometer (excitation λ = 490 nm; emission λ = 525 nm), that had been calibrated using a standard FITC solution (175 $ng.ml^{-1}$ of Tris/HCl buffer pH 8.5). One unit of proteinase activity was defined as the amount of enzyme causing a change of one RF unit h^{-1}.

(iii) Cleavage of β-casein by the crude proteinase

An aliquot (300 µl) of the crude proteinase preparation was added to 2 ml of 10 $mg.ml^{-1}$ β-casein and incubated at room temperature for 3 h. At the end of this time, the incubation mixture was ultrafiltrated using an Amicon Centricon microconcentrator with a molecular weight cut-off of 10 kDa to remove high Mr digestion products. The concentrate containing the proteinase and larger β-casein fragments was recovered and stored at -70 °C for recycling. The filtrate containing the β-casein peptides of interest was concentrated to 600 µl in a vacuum desiccater.

(iv) Separation and identification of the products of β-casein digestion

Samples (100-200 µl) of the β-casein digest were fractionated by HPLC on a reverse phase C18 column using a gradient of 0-40 % solvent B (0.08 % TFA in acetonitrile) over 40 min. Identification of the major peptide products was achieved by collecting each peak and sequencing by Edman degradation on an automated 470A Applied Biosystems Protein Sequencer with an on-line AB model 120A PTH analyser.
The obtained sequence for each peptide was then compared with the known amino acid sequence of β-casein.

(v) Cleavage of β-casein by purified H_2 proteinase and separation of the products

Digestion of β-casein by a purified proteinase from *L. cremoris* H_2 (supplied by Mr Julian Reid, Massey University) was also carried out using the same procedure as that described above for the proteinase from *L. cremoris* E_8.

(VI) Collection of β-casein oligopeptides

Three oligopeptides of interest were collected from repeated HPLC separations of the E_8 and H_2 digests of β-casein. Each of the three peptides were concentrated by repeated evaporation into the same eppendorf tube.

2.2.10 Peptide uptake experiments

(1) Peptide uptake experiments using the Val-$(Gly)_x$ peptide series.

The protocol used in these experiments was based on the work of Rice *et al.*, (1978). The following solutions were made up prior to the experiment.

(i) Buffer solution :

A phosphate buffer solution (pH 7.2) was prepared as follows

KH_2PO_4	90 mg
Na_2HPO_4	300 mg
distilled water	50 ml

The solution was filtered through a Millipore 0.22 µm filter and then sterilised in 6.5 ml aliquots at 15 psi for 15 min.

(ii) Glucose solution :
A 10 % (w/v) solution of glucose was prepared and sterilised at 15 psi for 15 min. Addition of 150 µl of this to 7.5 ml of incubation mixture gave a final concentration of approximately 0.2%.

(iii) Peptide solution :
A solution of the peptide of interest was made up so that when 1 ml was added to 6.5 ml of buffer solution it was at a final concentration of 1 mM. This was filtered through a 0.22 µm filter and sterilised at 15 psi for 15 min.

Milk-grown cultures of the strain of interest were grown through one passage of UBM and two passages of CDM in stbs. Once the culture in the second passage of CDM had reached an A_{540nm} of 1.5 - 2.0, the cells were harvested by centrifugation at 7000 g for 10 min at 4 °C. The cells were resuspended in cold 20 mM phosphate buffer and then recentrifuged under the same conditions.

(i) Peptide uptake by whole cells:

The washed cell pellet was resuspended in 6.5 ml of buffer solution which had previously been heated in a water bath to 30 °C. After 10 min to allow the cells to deplete endogenous energy sources, 150 µl of the glucose solution were added. After a further 15 min, 1 ml of peptide solution was added. Controls were also set up in which either the cells or the peptide were omitted.
Immediately after mixing by gentle swirling, a 200 µl sample was taken and added to 100 µl 3% TFA . The cells were precipitated by spinning at 13000 rpm in a MSE Microcentaur bench centrifuge. An aliquot (200 µl) of the resulting supernatant were removed and frozen until the sample could be analysed. This procedure was repeated at regular time intervals over 60 to 120 min.

During the experiment the pH was monitored to establish that the cells were actively metabolising the glucose.

A further 500 µl was removed from the incubation mixture after the zero time sample to determine cell dry weight. The absorbance of a suitably diluted volume of this sample was read at 540 nm. Cell dry weight of the sample was determined from dry weight versus A_{540nm}.

The addition of TFA to samples of the cell suspension was necessary to stop uptake immediately at the point of sampling, an advantage in some strains which transported peptides very rapidly. The TFA also prevented any leaked peptidases from degrading the peptide in timed samples when they were thawed for analysis.

A potential problem when adding the TFA before the cells were removed, rather than to the resulting cell-free supernatant, is that the disruption of the cells caused by the TFA could allow efflux of transported peptide back into the incubation buffer. This would distort any assessment of whole cell transport rates. However, previous work has shown that the lactococci hydrolyse transported peptides as rapidly as they move into the cell (van Boven and Konings, 1988) so the effect on supernatant peptide levels of adding TFA before the cells were removed should be minimal.
To demonstrate this experimentally, a comparative study was carried out to assess the effect of adding TFA to timed samples. In one set , TFA was added to samples before removing the cells by centrifugation. In the other set, cells were rapidly sedimented by centrifugation and TFA added to the supernatant. The results from this study (Figure 2.2.10.1) concur with the conclusion that the timing of addition of TFA has minimal effect upon the assessment of supernatant peptide levels. While supernatant Val-Gly_2 concentrations were on average about 10 percent lower in the timed samples where TFA was added after the cells were removed, compared to the samples where TFA was added before the cells were removed, this is consistent with the extra incubation time that occurred while the cells were being sedimented by centrifugation of these samples. However, the rate of peptide decrease was identical in both sample regimes.

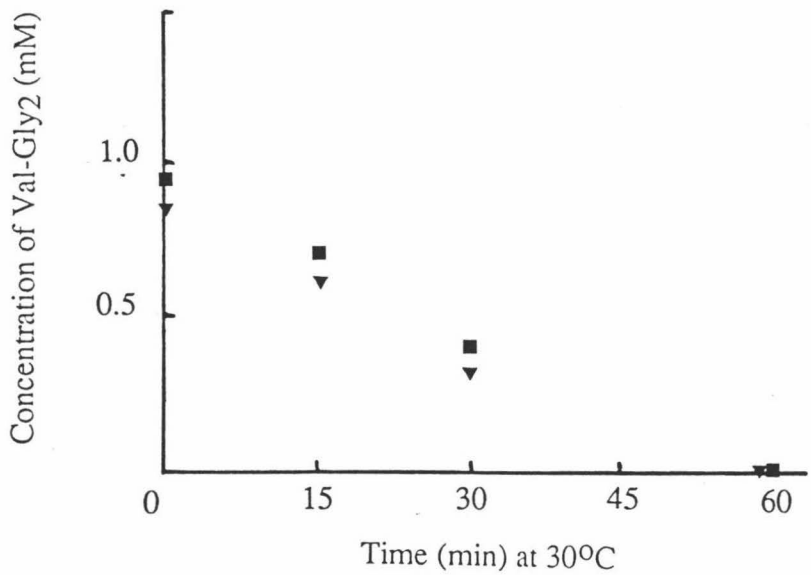


Figure 2.2.10.1 The effect of adding TFA to timed samples, either before the cells are removed (■), or after the cells are removed (▼), on the measurement of Val-Gly_2 uptake by cell suspensions of *L. cremoris* E8.

(ii) Peptidase control

In measuring peptide uptake , it was important to distinguish between peptide removal due to transport , and that due to the action of either extracellular peptidases, or leaked intracellular peptidases. The following procedure was used to determine the extent of peptide utilisation by peptidase activities in the medium.

At the end of 60 or 120 min, the cells were removed from the remaining incubation mixture. 1-3 ml of the supernatant were then filtered through a 0.22 μm filter. Peptide solution was added to this filtrate to give a final concentration of 1 mM, assuming all the original peptide had been metabolised by the cells. A 100 μl sample was removed immediately after mixing and added to 50 μl of 3 % TFA. Further samples were taken at regular intervals over a 60 min interval. These were then stored at -20 °C for the subsequent determination of the rate of peptide hydrolysis.

(iii) Analysis of samples by capillary electrophoresis.

The peptide concentration in samples of supernatant from the peptide uptake experiments, and the peptidase controls, was determined by capillary electrophoresis (CE).Thawed samples were filtered through a 0.22 μm filter and 40 μl of the filtrate pipetted into CE minieppendorfs. The peptide content of these samples was then determined on an ABI High Throughput Capillary Electrophoretic System, model 270A-HT, with a 72 cm length fused silica column (50 μm internal diameter; 360 μm external diameter; 20 μm thickness).

The print-out generated by the CE for each time sample reports the integrated areas of the major peptide peaks separated. Each peak of interest was quantified by expressing its area as a percentage of the area of the same peptide peak in the zero time sample. Theoretically the area of the zero time peak of the peptide should be 1 mM. However this assumes that all of the Val-Gly_x peptides synthesised were 100 percent pure. To estimate the percentage purity of the synthesised peptides, it was assumed that the absorbance at 220 nm for each peptide was attributable solely to the peptide bond, and that the A_{220} for each peptide would increase in linear proportion to the number of peptide bonds. Since the commercially supplied Val-Gly_2 was reported to be 98 % pure by chromatographic analysis, this was used as a standard. The experimentally determined peak areas for the synthesised Val-Gly_x peptides were compared with the

theoretically predicted areas. The latter were calculated on the basis of the Val-Gly_2 peak area and the above assumption concerning the relationship between the A_{200} and the number of peptide bonds.

This assumption was shown to be valid by comparing the experimentally found areas of 1 mM solutions of Sigma quality di-L-alanine, tri-L-alanine, tetra-L-alanine, penta-L-alanine and hexa-L-alanine, with their expected areas based on that of dialanine alone (Table 2.1.10.1).

Table 2.2.10.1 Comparison of the experimentally determined capillary electrophoresis areas of 1 mM solutions of di-L-alanine, tri-L-alanine, tetra-L-alanine, penta-L-alanine and hexa-L-alanine , with their expected areas relative to that of di-L-alanine.

Peptide (1 mM)	Number of peptide bonds	Expected area relative to Ala-Ala.	Experimentally found CE area.
di- L-alanine	1	43, 622.5	43, 622.54
tri-L-alanine	2	87, 245.0	88, 594.41
tetra-L-alanine	3	130, 867.5	136, 290.25
penta-L-alanine	4	174, 490.0	176, 358.30
hexa-L-alanine	5	218, 112.5	225, 933.0

(iv) Calculation of the rate of peptide uptake

The rate of decrease in peptide concentration in the supernatant from the cell suspension was calculated over the 60 or 120 min that the experiment ran for. Similarly, the rate of removal of peptide due to any peptidase activity was also calculated, as described above.

The rate of peptide uptake by whole cells was determined from these two values by subtracting the peptidase rate from the peptide removal rate. Determination of cell dry

weight from a separate sample of the suspension enabled the rates to be expressed as nmol min^{-1} (mg dry weight)$^{-1}$. Detailed calculations are shown in Section 4.1.3.3

(2) Peptide uptake experiments using the β-casein oligopeptides.

Prior to the experiment, each of the frozen stocks of the three oligopeptides purified from HPLC separations of a proteinase digest of β-casein (Section 2.2.9) were redissolved in 500 µl of distilled -deionised water.

Milk-grown cells of *L. cremoris* E8 were grown through one passage of UBM and two passages of CDM in stbs. Once the culture from this second passage had reached an A_{540nm} of 1.5 - 2.0 the cells were harvested and washed as described in Section 2.2.10. The washed cell pellet obtained was resuspended in incubation buffer (100 mM MES/ 50 mM glucose/ 10 mM $MgSO_4$) to a concentration that when diluted 100-fold gave an A_{540nm} of approximately 0.35. This cell-buffer mixture was then used to set up the following three incubation systems in eppendorf tubes;

(a) 300 µl cells/buffer + 300 µl of peak 1 (KAVPYPQ)

(b) 300 µl cells/buffer + 300 µl of peak 2. (QEPVLGPVRGPFPIIV)

(c) 300 µl cells/buffer + 300 µl of peak 3 (RDMPIQAFLLY)

Each cell/ buffer/ peptide system was incubated at 30 °C. Immediately after mixing a 75 µl aliquot sample was taken and mixed with 25 µl of 3 % TFA. After spinning in a bench microcentrifuge to precipitate the cells, a 50 µl aliquot was removed and frozen until it could be analysed. This procedure was repeated at regular intervals for up to 60 min.
The peptide concentration of these samples was determined using capillary electrophoresis as described in Section 2.2.9(iii).

2.2.11 Isolation of a lactococcal mutant defective in dipeptide utilisation

(i) Synthesis of L-alanyl-β-chloroalanine(AβClA)

The procedure used in attempts to isolate a mutant unable to utilise dipeptides was developed by Smid *et al.*, (1989b). This procedure is based on the toxicity of free β-chloroalanine to lactococci. A dipeptide containing β-chloroalanine will be toxic only when degraded to the free amino acids, so providing a positive selection method for isolating mutants deficient in their ability to transport and/or to hydrolyse a particular peptide (see Chapter 5).

The chemical structure of the β-chloroalanine containing dipeptide used in this present study is shown in Figure 2.2.11.1.

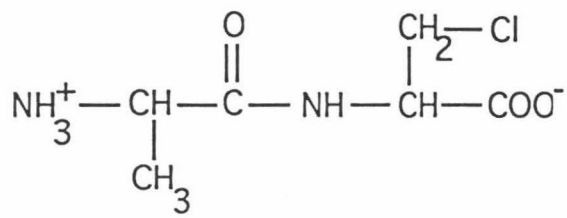
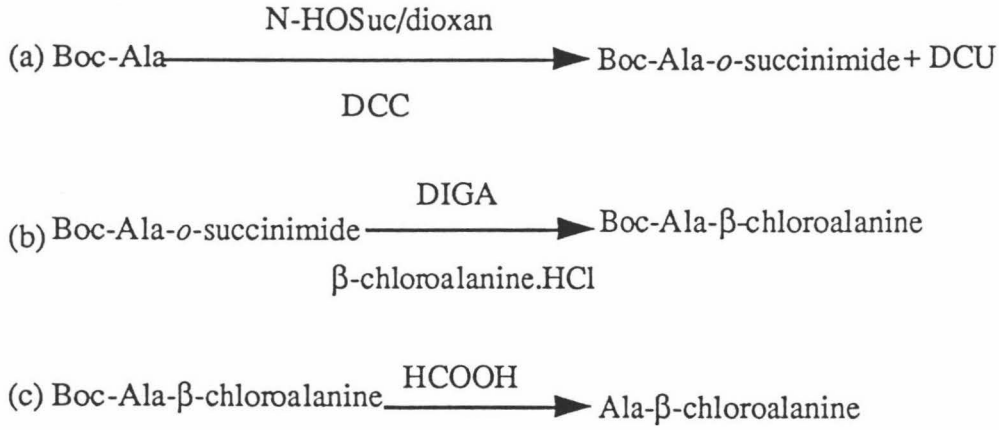


Figure 2.2.11.1 Structure of L-alanyl-β-chloroalanine

This peptide was synthesised using a solution phase activated ester method summarised as follows.



To 1.69 x 10^{-3} mol of Boc-alanine (Boc-Ala), 3.38 x 10^{-3} mol of N-hydroxysuccinimide (N-HOSuc) were added. Dioxan (15 ml) was added and the mixture left to stir over ice for 15 min. At the end of this time, 1.69 x 10^{-3} mol of dicyclohexylcarbodiimide (DCC) were added and the mixture left to stir at room

temperature for 3 h. The dicyclohexylurea (DCU) produced by the end of this time was filtered off to leave a clear solution.
The HCl salt of β-chloroalanine (1.88×10^{-3} mol) was separately dissolved in 3.76×10^{-3} mol of the hindered base di-isopropyl ethylamine (DIEA), and 2 ml of di-methyl formamide (DMF). A hindered base was chosen as it should not attack the chloride substituent of the alanyl methyl group. This solution was mixed with that containing the Boc-ala-*o*-succinimide until the mixture cleared (approximately 6 h). The DMF was removed by evaporation and the residue was freeze-dried overnight. The Boc group was then removed from the peptide and further purification carried out as described for Val-Gly_3.(Section 2.2.8.(i)).

(ii) Identification of the peptide.

The resulting crystals proved to be very insoluble in aqueous media, which created problems for getting a sample for analysis and identification. The crude peptide was sonicated in water and an equilibrium allowed to develop between the dissolved and undissolved states. The residue precipitate was filtered off and a sample of the filtrate applied to an HPLC C18 analytical column (see Section 5.1 for details).
Peaks from a gradient separation of these filtrates believed to correspond to L-alanyl-β-chloroalanine were collected and sent for molecular weight determination by SIMS mass spectrometry (Section 5.2).

(iii) Use of L-alanyl-β-chloroalanine to isolate a possible mutant

Once confirmation had been obtained that the crude crystals contained L-alanyl-β-chloroalanine (AβClA) an attempt was made to use this peptide in the selection of mutants unable to degrade the peptide to the toxic β-chloroalanine (DiP^- mutants)
A source of AβClA was placed on a growing lawn of cells. The potentially toxic peptide should diffuse out into the surrounding medium and form a concentration gradient in which only spontaneous mutants unable to degrade this peptide will grow. Completely defined medium containing 1.5 % agar was prepared and sterilised by autoclaving. Appropriate quantities of lactose solution and vitamin-mineral solution (Section 2.1.1) were aseptically added after autoclaving and before setting and pouring Petri plates of this medium.
The experiments to isolate a dipeptide-utilising mutant were initiated by growing

L. cremoris E8 through two passages of CDM solution in screw-topped bottles. A 50 µl aliquot of cells from the second passage in CDM was spread onto CDM plates with a flamed glass rod. These plates were then incubated at 30 °C for 4 h to allow for the initiation of a bacterial lawn.

One of two approaches was then employed in applying the toxic dipeptide to the agar plates to select for mutants in these bacterial lawns.

(i) several of the crude crystals containing AβClA were placed directly onto the plates.

(ii) samples of purified AβClA were pipetted into aseptically cut wells in the agar (Figure 2.2.11.2). AβClA was separated from contaminants in the crude crystals by carrying out HPLC separations of aqueous extracts of these crystals, as described above in detail. The peak corresponding to AβClA was collected from multiple separations and concentrated. A control well containing 1 % TFA was included to check whether growth inhibition may have been due to residual TFA in the purified AβClA.

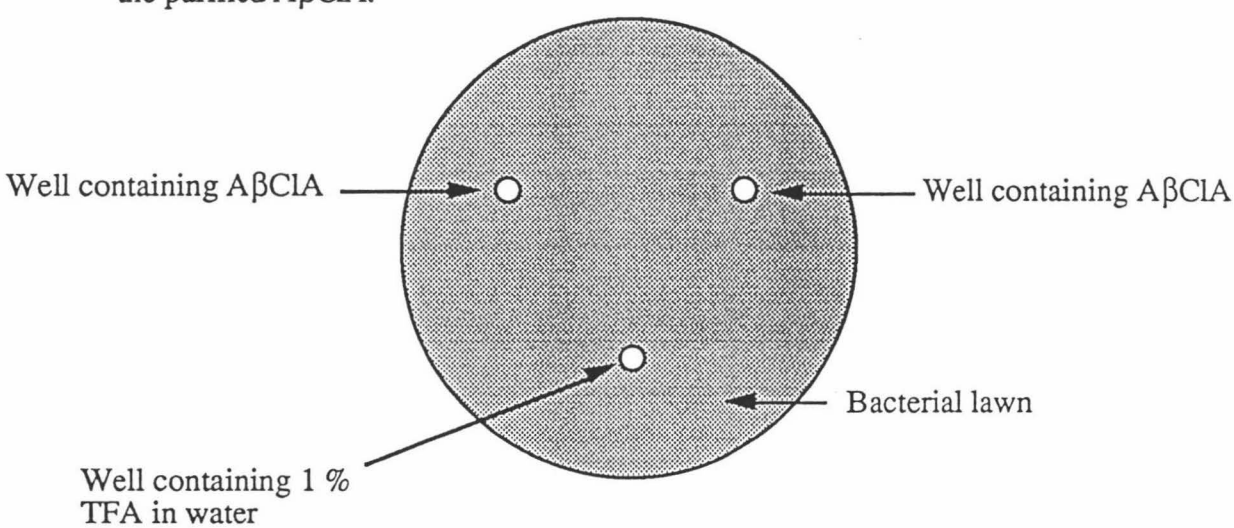


Figure 2.2.11.2 Diagrammatic representation of a method employed to use alanyl-β-chloroalanine to select for mutants deficient in their ability to transport and/or hydrolyse dipeptides. Samples of HPLC purified peptide were pipetted into wells on a Petri dish containing CDM on which was growing a 4 h old culture of strain E8. As a control, a sample of water containing 1 % TFA was pipetted into a separate well.

In either case, the plates were incubated at 30 °C for 24-36 h. At the end of this time they were observed for zones of inhibition around the sources of toxic dipeptide. Any colonies growing within these zones were streaked out initially onto CDM + Val plates, which provided nutrients in a form that bypasses the mutation. When colonies of each possible mutant had grown up to sufficient size on these plates, they were subcultured onto CDM-Val, CDM + Val, CDM + Val-Gly, and CDM + Val-Gly_2 plates to identify the nature of the mutation. A mutant deficient in the di-/tripeptide transport system would not be expected to grow on Val-Gly or Val-Gly_2 as sources of the essential amino acid valine. A mutant deficient in dipeptidase activity would not be expected to grow on chemically defined medium supplemented with Val-Gly, but should be capable of growth in defined medium where valine is supplied as the tripeptide Val-Gly_2.

Chapter Three - Characterisation of peptides.

3.1 Purification and characterisation of synthetic peptides

A homologous series of Val-$(Gly)_x$ peptides was synthesised using either solution phase or solid phase peptide synthesis chemistry. Valine was chosen as the amino acid on which to base this series for two reasons ;

(i) Valine is essential for the growth of both *L. lactis* strains and *L. cremoris* strains.

(ii) The peptides Val-Gly and Val-Gly_2 are commercially available from Sigma at reasonable prices.

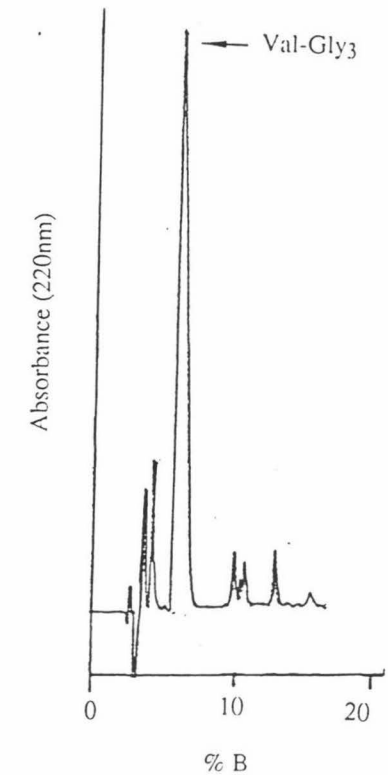
3.1.1 Val-Gly_3

This tetrapeptide was synthesised by solution phase chemistry as detailed in section 2.2.8 (i). An HPLC profile of the crude product from this synthesis is shown in Figure 3.1.1.1 (a). As it was intended to use the Val-$(Gly)_x$ peptides for growth and transport studies, it was important to purify them from contaminants which could influence the results of such studies. This was achieved by repeated isocratic separations of the crude product on a Synchroprep preparative HPLC C18 column as detailed in section 2.2.8 (i). HPLC analysis of the fractions collected showed that the Val-Gly_3 peptide eluted early in the separation profile, while the major contaminants were retained on the column (data not shown). An HPLC profile of the peptide purified by this separation is shown in Figure 3.1.1.1(b).

Two procedures were carried out to confirm the identity of this peptide.

Amino acid analysis of a hydrolysed sample gave a Val : Gly ratio of 1 : 3.3 which is in good agreement with the expected ratio for Val-Gly_3 of 1 : 3 (Table 3.1.1.1). The absence of any other major amino acid peaks also attests to the purity of this peptide.

A sample subject to mass determination by SIMS gave a strong peak at a mass of 289 which is the expected mass of the MH^+ ion of Val-Gly_3 (Figure 3.1.1.2).



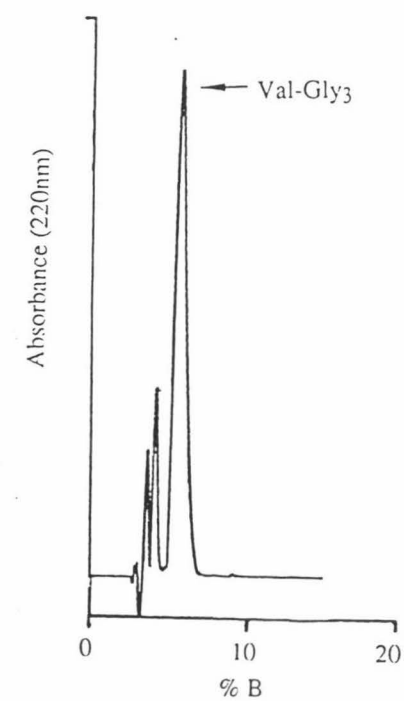
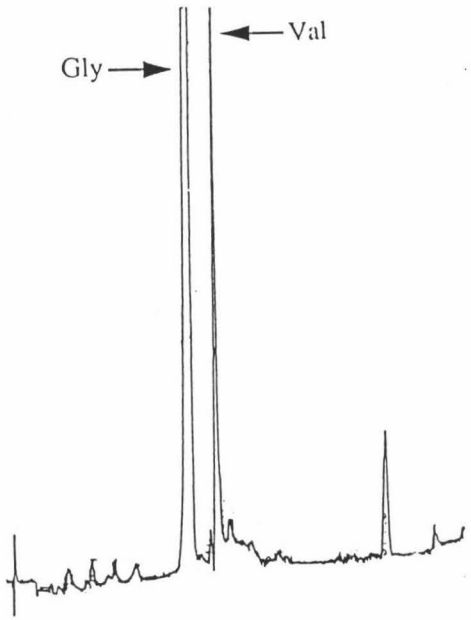


Figure 3.1.1.1 Reverse phase HPLC profiles of (a) the crude peptide product resulting from the solution phase synthesis of Val-Gly3, and (b) the purified product after passage through a C18 preparative column. Solvent A was 0.1 % TFA in water, and solvent B was 0.08 % TFA in acetonitrile. The products were eluted with a linear gradient of 0-15 % B over 15 min.



Amino Acid	nmol.100µl^{-1}	Ratio of Val:Gly
Val	12.38	1.0
Gly	41.33	3.3

Table 3.1.1.1 Amino acid analysis of the purified product obtained from Val-Gly3 synthesis

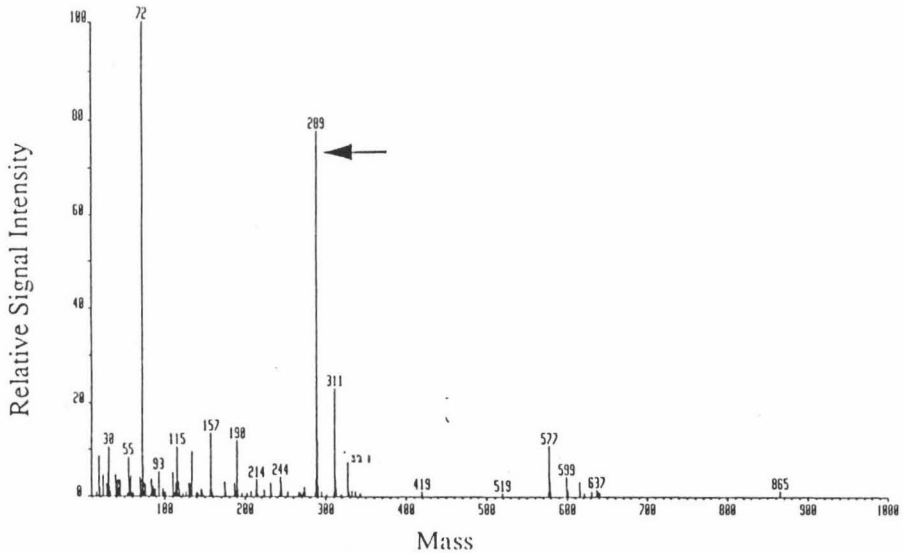


Figure 3.1.1.2 Mass spectrum of the purified product from Val-Gly3 synthesis. The horizontal arrow (←) marks the mass peak corresponding to the expected mass of the MH^+ ion of Val-Gly3.

3.1.2 Val-Gly_4

This pentapeptide was synthesised by solid phase peptide chemistry as detailed in Section 2.2.8 (ii). An HPLC profile of the crude peptide is shown in Figure 3.1.2.1 (a). This was significantly purified by repeated isocratic separation on an HPLC Synchroprep C18 preparative column (Figure 3.1.2.1(b)).

Two procedures were carried out to confirm the identity of the major HPLC peak.

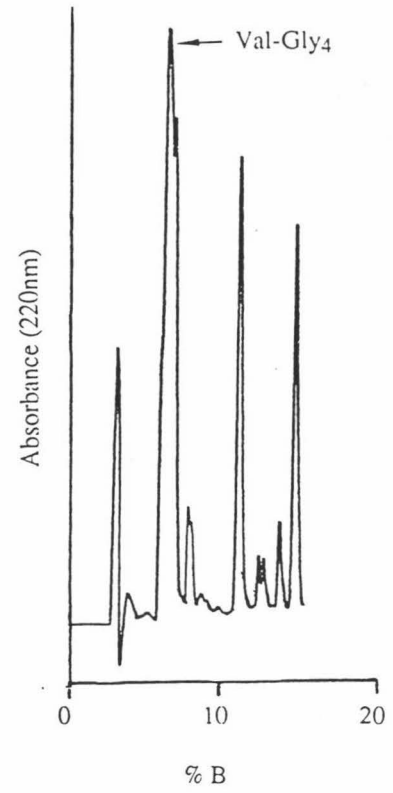
Amino acid analysis of a hydrolysed sample gave a Val : Gly ratio of 1 : 3.6 which is in reasonable agreement with the expected ratio for Val-Gly_4 of 1 : 4 (Table 3.1.2.2).

Mass determination gave a strong peak at a mass of 346, the expected value of the MH^+ on of Val-Gly_4 . However peaks could also be seen at mass values of 289 and 232 which correspond to the MH^+ ions of Val-Gly_3 and Val-Gly_2 respectively. (Figure 3.1.2.2). The presence of these smaller peptides was confirmed when a sample of the purified Val-Gly_4 was analysed by capillary electrophoresis (Figure 3.1.2.3.).
This mixture of peptides is the result of incomplete coupling during synthesis. This event often occurs when the solid support resin begins to degrade. The similar chemical nature of these peptides causes them to co-elute in HPLC analysis, making it impossible to use reverse phase HPLC to purify the Val-Gly_4 further. While capillary electrophoresis (CE) does separate Val-Gly_4 from these smaller peptides (Figure 3.1.2.3), this technique is incapable of dealing with usable volumes of the peptide for preparative work.

Ideally it would have been preferable to resynthesise Val-Gly_4. However this would have necessitated constructing a new resin for the solid phase synthesis. This was not possible due to the work commitments of the Massey University SSU. Since the level of contaminants in the Val-Gly_4 preparation was relatively low, it was considered that they would not significantly effect the interpretation of results in the experiments envisaged using this peptide.

3.1.3 Val-Gly_7

An HPLC elution profile of the purified peptide supplied by Massey University SSU is shown in Figure 3.1.3.1.



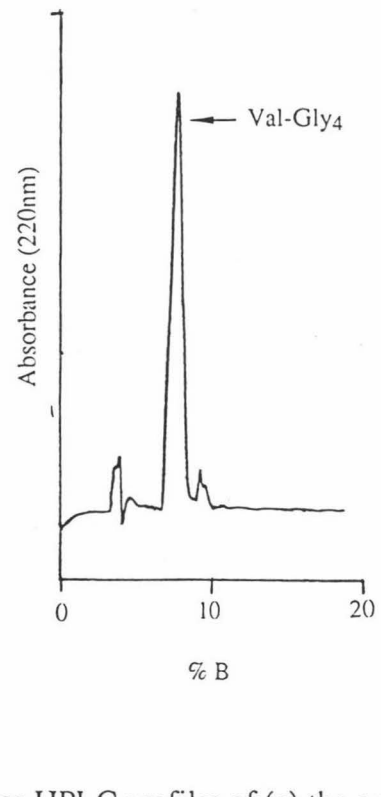
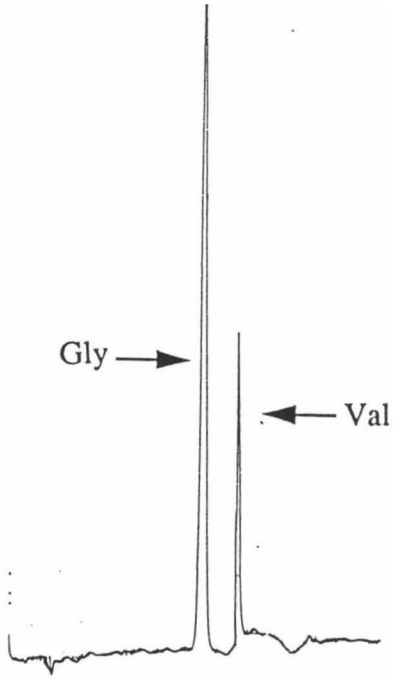


Figure 3.1.2.1 Reverse phase HPLC profiles of (a) the crude peptide product resulting from the solution phase synthesis of Val-Gly4, and (b) the purified product after passage through a C18 preparative column. Solvent A was 0.1 % TFA in water, and solvent B was 0.08 % TFA in acetonitrile. The products were eluted with a linear gradient of 0-15 % B over 15 min.



Amino Acid	nmol.100µl^{-1}	Ratio of Val:Gly
Val	12.84	1.0
Gly	46.54	3.6

Table 3.1.2.1 Amino acid analysis of the purified product obtained from Val-Gly4 synthesis.

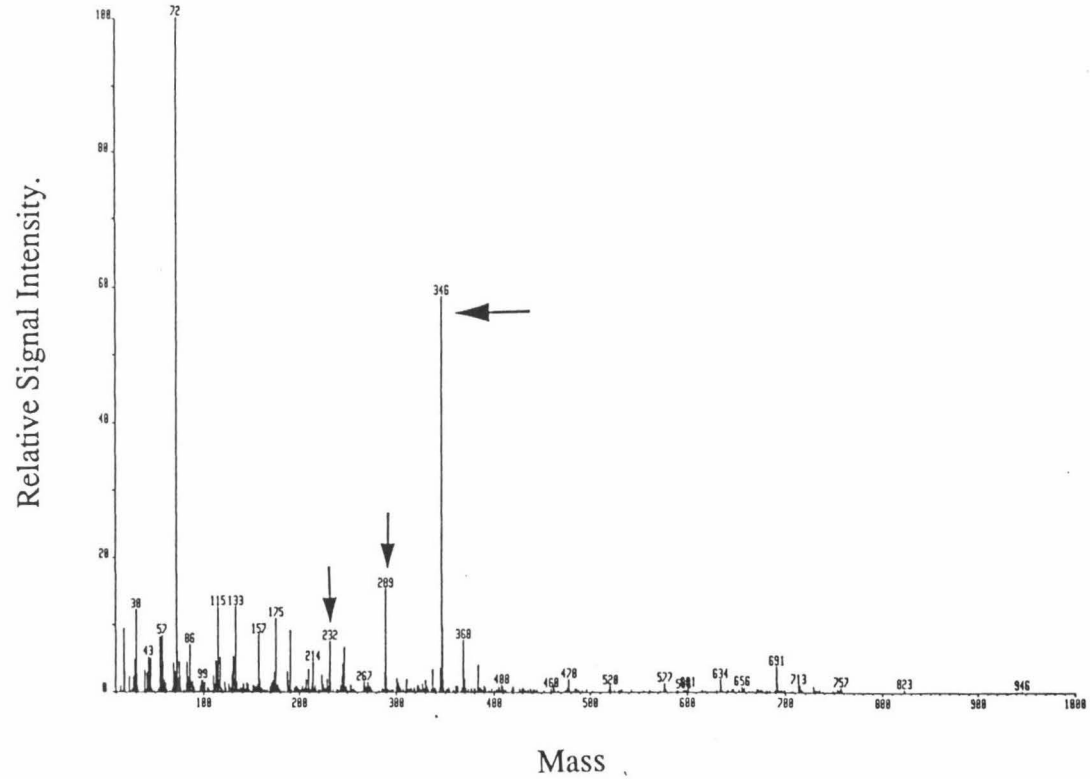


Figure 3.1.2.2 Mass spectrum of the purified product from Val-Gly4 synthesis. The horizontal arrow (←) marks the peak corresponding to the expected mass of the MH^+ ion of Val-Gly4. The vertical arrows (↓) marking peaks at mass values of 289 and 232 correspond to Val-Gly3 and Val-Gly2 respectively.

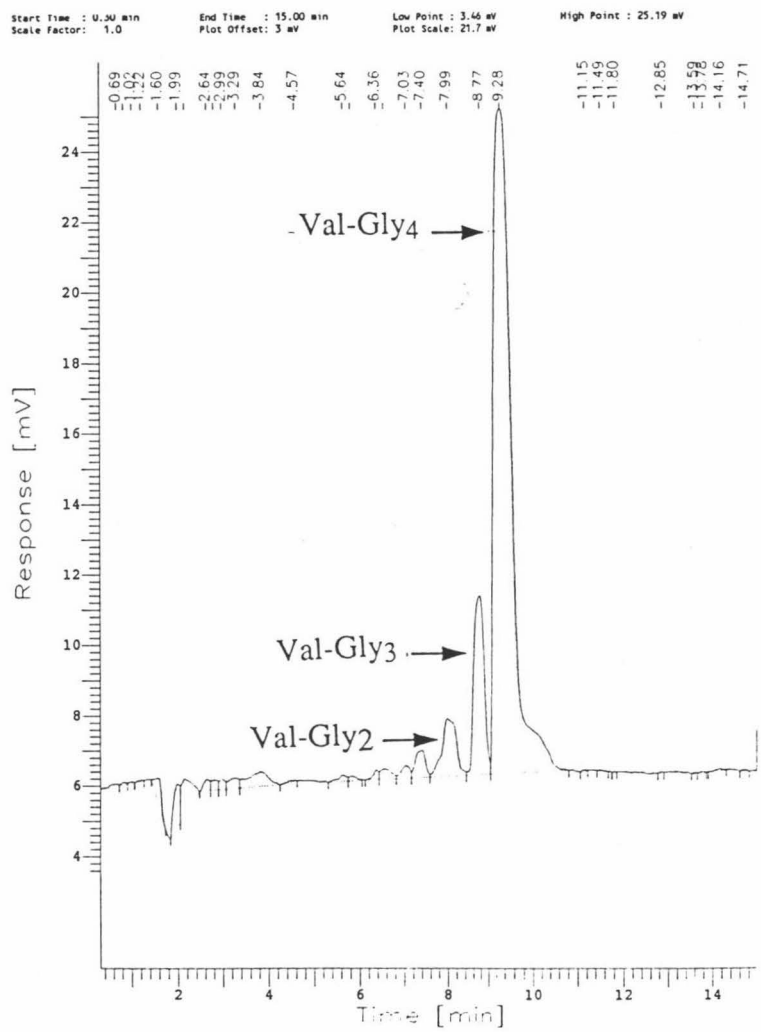


Figure 3.1.2.3 Capillary electrophoresis profile of the purified product obtained from Val-Gly4 synthesis. A 2.5 sec injection was made of the sample onto a 72 cm Freezone column. The sample was separated by running in 20 mM sodium citrate, pH 2.5 at 30 kV for 15 min.

Amino acid analysis of this peak gave a Val : Gly ratio of 1 : 7.2, which is in good agreement with the expected ratio for this peptide of 1 : 7.
(Table 3.1.3.2).

Molecular weight determination by SIMS gave a major peak at 517, which is the expected mass of the MH^+ ion of Val-Gly_7 (Figure 3.1.3.2).

3.1.4 Val-Gly_9

The decapeptide Val-Gly_9 proved very difficult to synthesise in any reasonable amount. An HPLC profile of the purified product supplied to the author shows two major products, the second of which was identified from amino acid analysis to be Val-Gly_9.(Figure 3.1.4.1). Due to the minimal amount of peptide available for use, it was decided not to risk loss of yield by attempting to purify the decapeptide further.

Amino acid analysis of the Val-Gly_9 peak gave a ratio of 1 : 9.9 which is in reasonable agreement with the expected value of 1 : 9. (Table 3.1.4.2).

The mass analysis however was inconclusive. Although a peak corresponding to the expected MH^+ ion of 631 was present, it was not much higher than the background noise (Figure 3.1.4.2). It was decided after several unsuccessful repeat attempts that this result may have been due to a failure of the Val-Gly_9 to "fly" from the glycerol solvent used, rather than to insignificant levels of peptide. This assumption is supported by the strong signals obtained during HPLC analysis and amino acid analysis.

To obtain further evidence that the synthesised peptide was Val-Gly_9, N-terminal sequencing was carried out. This analytical technique is limited in its usefulness when there is a long sequence of identical amino acid residues; in this case nine sequential glycines. The presence of a continued strong signal for glycine after 12 sequencing cycles, does however indicate that the peptide may have been around 10 residues long (data not shown). Furthermore the mass profile for Val-Gly_9 does not appear to contain mass peaks corresponding to smaller Val-$(Gly)_x$ peptides.
However , in view of the uncertainty concerning the homogeneity and identity of the decapeptide, it was decided not to use it in growth and uptake studies.

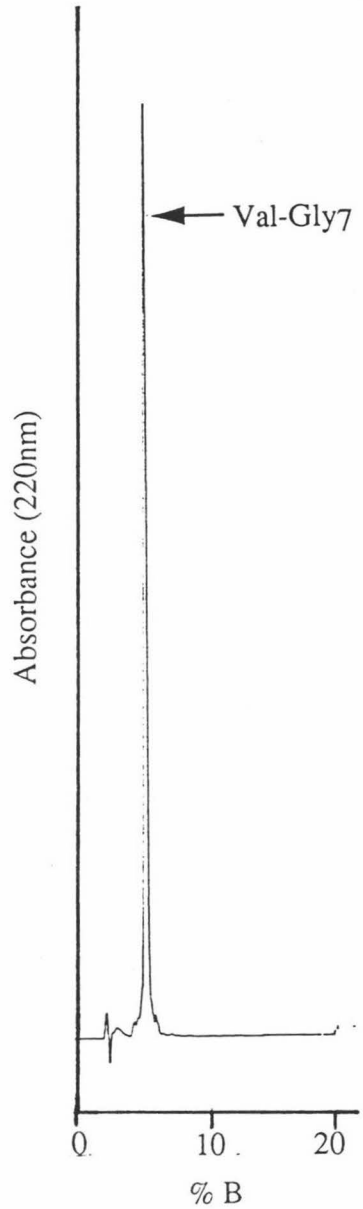
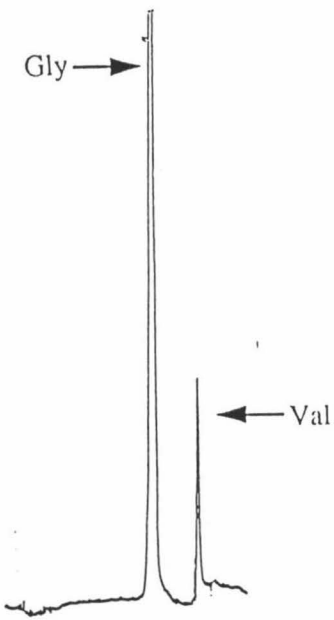


Figure 3.1.3.1 Reverse phase HPLC profile of the purified product from the solid phase peptide synthesis of Val-Gly7. Solvent A was 0.1 % TFA in water, and Solvent B was 0.08 % TFA in acetonitrile. The products were eluted with a linear gradient of 0-15 % B over 15 min.



Amino Acid	nmol.100µl^{-1}	Ratio of Val:Gly
Val	22.03	1.0
Gly	158.49	7.2

Table 3.1.3.1 Amino acid analysis of the purified product obtained from Val-Gly7 synthesis

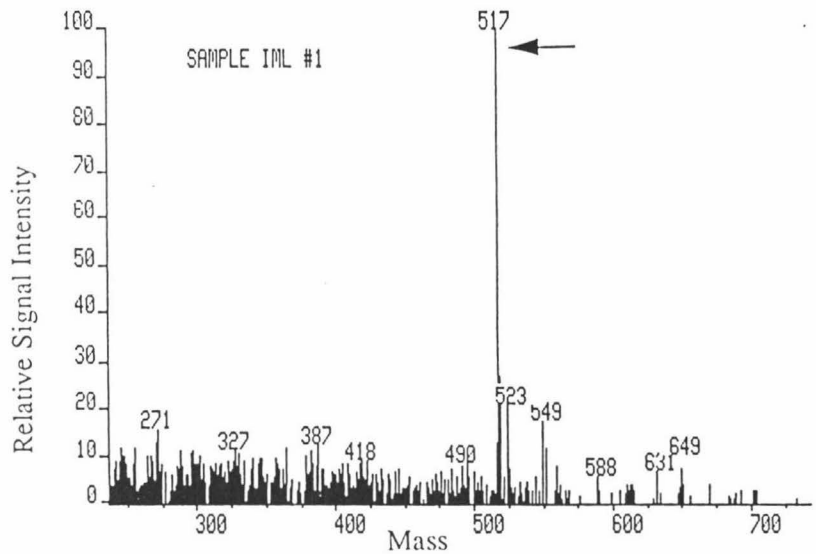


Figure 3.1.3.2 Mass spectrum of the purified product from Val-Gly7 synthesis. The horizontal arrow (←) marks the mass peak corresponding to the expected mass of the MH^+ ion of Val-Gly7.

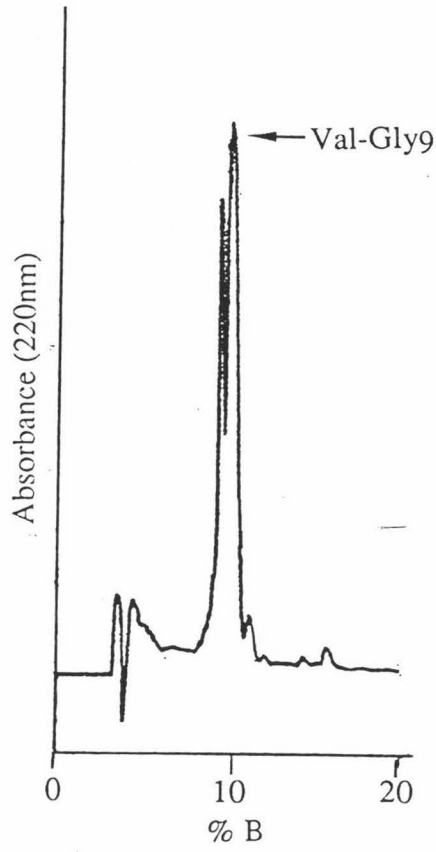
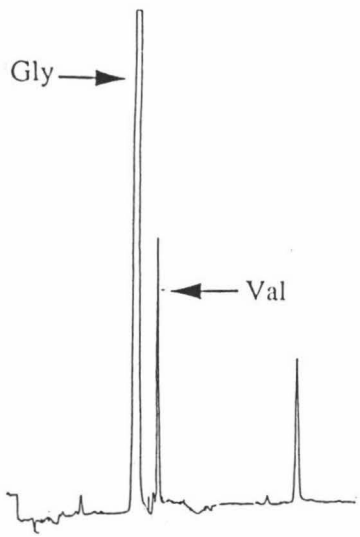


Figure 3.1.4.1 Reverse phase HPLC profile of the purified product from the solid phase peptide synthesis of Val-Gly9. Solvent A was 0.1 % TFA in water, and Solvent B was 0.08 % TFA in acetonitrile. The products were eluted with a linear gradient of 0-15 % B over 15 min.



Amino Acid	nmol.100µl^{-1}	Ratio of Val:Gly
Val	18.57	1.0
Gly	183.73	9.9

Table 3.1.4.1 Amino acid analysis of the purified product obtained from Val-Gly9 synthesis.

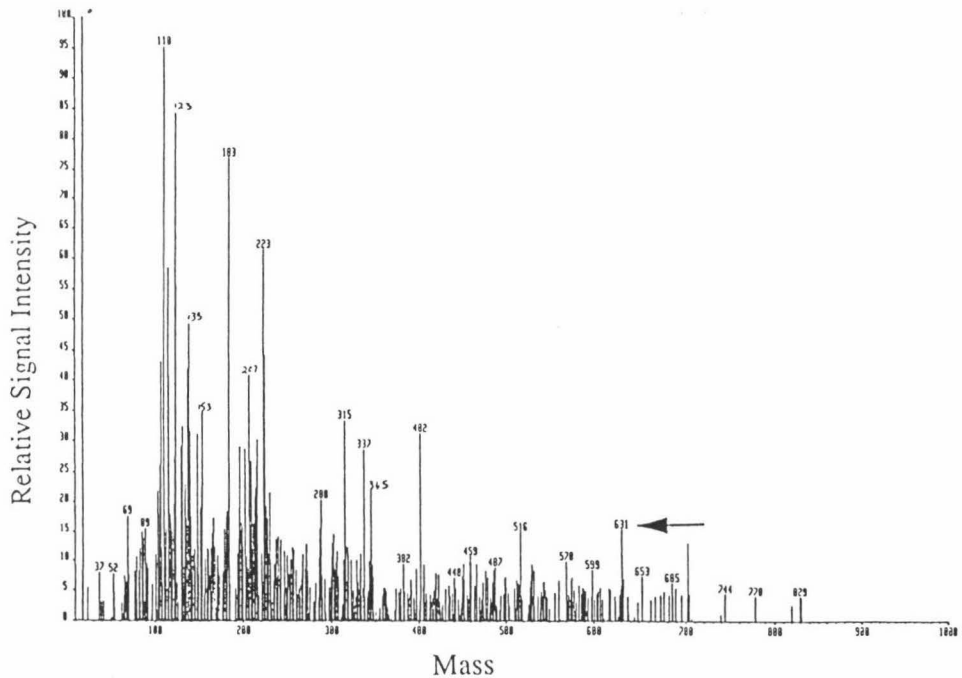


Figure 3.1.4..2 Mass spectrum of the purified product from Val-Gly9 synthesis. The horizontal arrow (←—) marks the peak corresponding to the expected mass of the MH^+ ion of Val-Gly9.

3.2 Isolation and characterisation of β-casein peptides.

In addition to using a synthetic peptide series, peptides produced by the action of the cell wall-associated proteinase on β-casein were used to study peptide uptake. These milk peptides were isolated by carrying out a proteinase digest of β-casein and purifying products of that digestion

3.2.1 Fractionation of the peptide products from the β-casein digestion

A crude proteinase extract was prepared from milk-grown *L. cremoris* E8 cells (Section 2.2.9). HPLC separation of a 3 h digest of β-casein by this crude extract produced a complicated profile (data not shown). N-terminal sequencing of the major peaks in this HPLC profile showed that most contained more than one peptide, and that many of these peptides were truncated due to the activity of contaminating peptidases in the proteinase extract. These features made this digest unsuitable as a source of substrates for the planned transport experiments .

Although the initial cleavage pattern of β-casein by the E8 proteinase was complicated by the presence of peptidases in the crude extract, it could be seen to be more typical of a P_I-type proteinase than a P_{III}- type proteinase.
It was therefore decided to use purified proteinase from *L. cremoris* H_2, which was available in the laboratory, as an alternative source of the desired β-casein derived peptides. The proteinase of *L. cremoris* H_2 is a typical P_I-type, and produces a simple initial cleavage pattern of β-casein when analysed by reverse phase HPLC (Figure 3.2.1.1). The work of Reid *et al*. (1991a) has identified the amino acid sequences of the three major peaks (Table 3.2.1.1) and these align with the C-terminal end of β-casein (Figure 3.2.1.2).

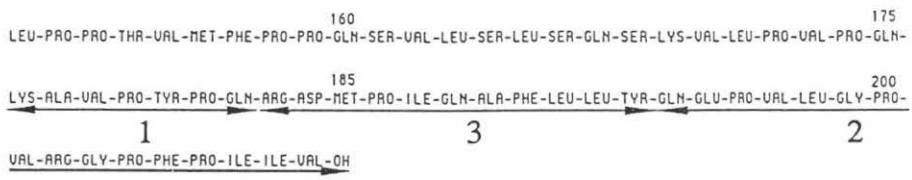


Figure 3.2.1.2 Alignment of the peptides produced from β-casein hydrolysis by a purified extract of the cell envelope-associated proteinase of *L. cremoris* H_2 against the known sequence of the C-terminal end of β-casein. Peptides are labelled according to their HPLC peaks of origin in Figure 3.2.1.1.

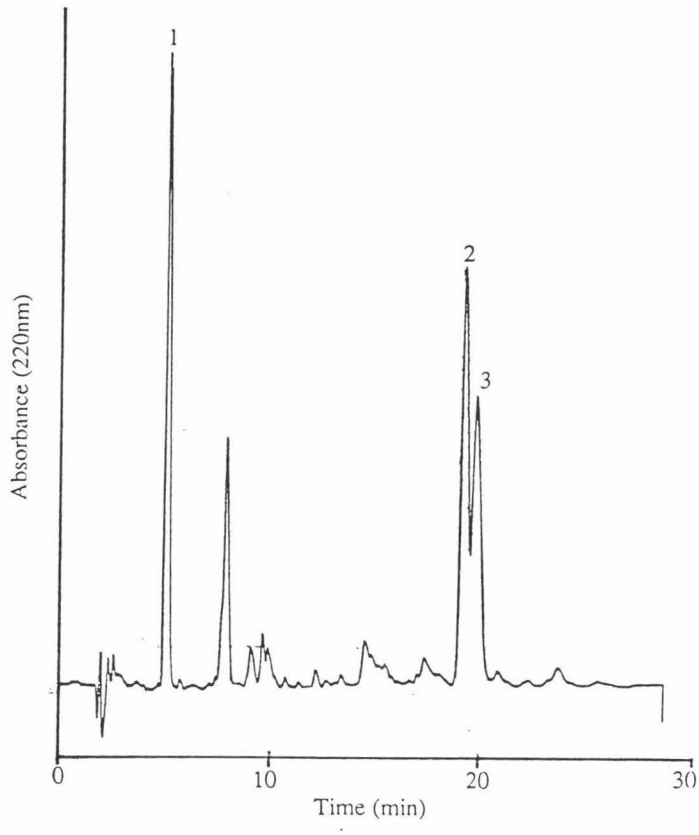


Figure 3.2.1.1 Reverse phase HPLC profile of a 3 h digest of β-casein by a purified extract of the cell envelope-associated proteinase from *L. cremoris* H_2. The three peaks that were collected for sequencing are labelled. Solvent A was 0.1 % TFA in water, and Solvent B was 0.08 % TFA in acetonitrile. The products were eluted with a linear gradient of 0-40 % B over 40 min.

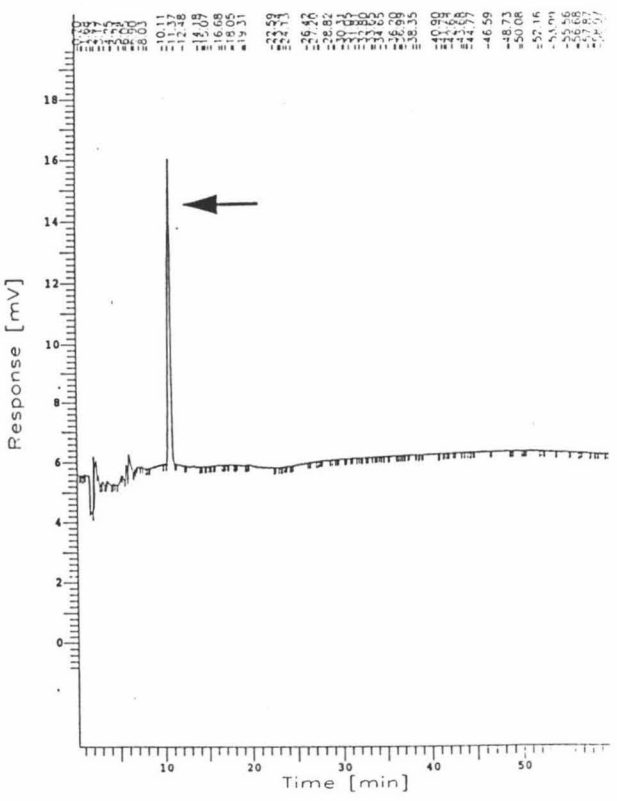
Peak	Peptide Sequence	Position in β-casein
1	KAVPYPQ	176 - 182
2	QEPVLGPVRGPFPIIV	194 - 209
3	RDMPIQAFLLY	183 - 193

Table 3.2.1.1 Amino acid sequences of peptides detected within peaks from an HPLC separation of a β-casein hydrolysate by a purified extract of the cell envelope-associated proteinase of *L. cremoris* H_2.

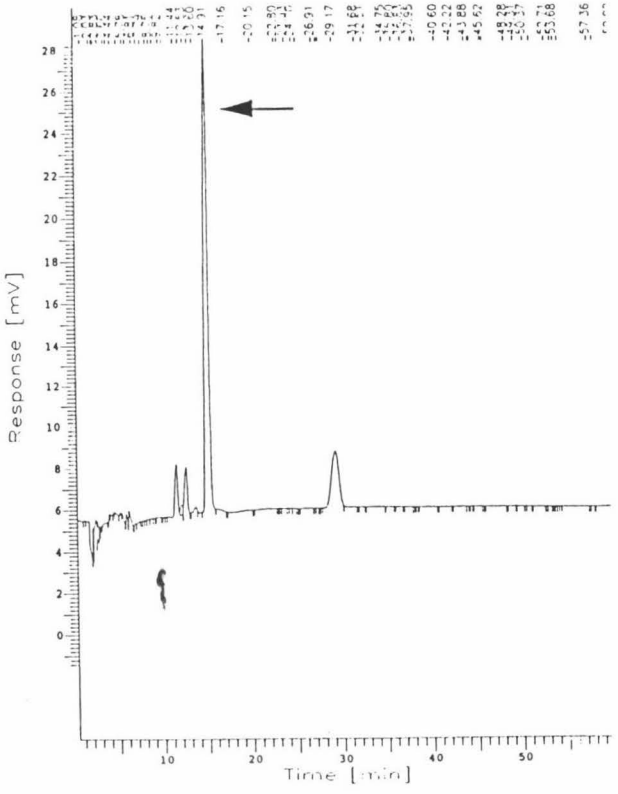
As all three peaks contain only one peptide product they are ideal sources of substrates for the transport experiments.

3.2.2 Confirmation of peptide purity

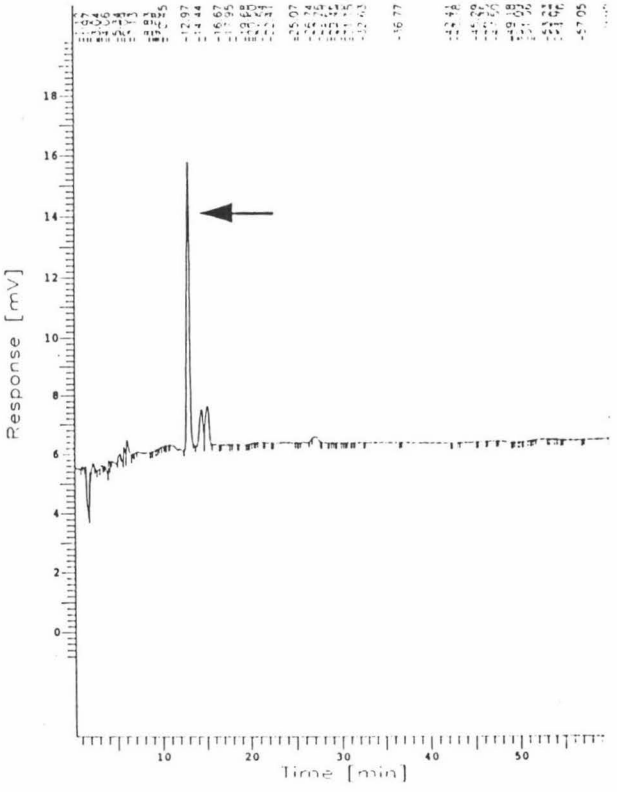
After repeated HPLC fractionation to obtain usable quantities of the three peptides detailed in Table 3.2.1.1, each was assessed for purity by capillary electrophoresis (Figure 3.2.2.1). Peptide (1) was found to be completely pure, whereas peptides (2) and (3) had minor contaminants



Peptide (1) : KAVPYPQ



Peptide (2) : QEPVLGPVRGPFPIIV



Peptide (3) : RDMPIQAFLLY

Figure 3.2.3.1 Capillary electrophoresis profiles of each of the three major peptides produced from β-casein hydrolysis by the cell wall-associated proteinase of *L. cremoris* H_2. The arrows mark the position of each peptide in its CE profile. A 2.5 sec injection was made of the sample onto a 72 cm Freezone column. The sample was separated by running in 20 mM sodium citrate, pH 2.5 at 30 kV for 15 min.

Chapter Four - Peptide utilisation by lactococci

As defined earlier (Section 1. 5), the central aim of this research was to investigate aspects of peptide utilisation by certain *L. lactis* and *L. cremoris* strains. Early work (Law, 1977;1978) had indicated significant differences in peptide utilisation between strains (Section 1.4.2). Yet most of our current knowledge regarding the mechanisms of peptide transport is drawn from generalisations based on the intensive study of only one strain. Comparative studies on a range of strains were carried out to test whether these generalisations are valid.

Initial investigations in the present study were centred on *L. lactis* 1403, which proved easy to grow on a chemically defined medium. Other strains would grow in this medium, but only after very long lag phases in the order of 8 - 12 hours (These lag phases were later discovered to be a result of the physiological state of the cells used to inoculate the medium. They could be prevented by more frequent transfers to ensure that the cultures used to inoculate the medium were in an actively growing state).

Using strain 1403, growth on a range of complex and defined media, which supplied essential amino acids in a variety of forms, was investigated. The activity of several different peptidases which may contribute to peptide utilisation was determined for cells grown on each of these media to assess whether the peptidase complement is regulated by the nature of the peptide source.
Subsequent studies to investigate peptide uptake by this strain revealed a major problem leading us to select other lactococcal strains to pursue further work on peptide utilisation. (see Sections 4.1.3 and 4.1.4)

Further studies with whole cells were carried out on two other strains, *L. cremoris* E8 and *L. lactis* 920. The following experiments were carried out in one or both of these strains.

(a) Studies on peptide uptake using the synthesised Val-$(Gly)_x$ series.

(b) Studies on growth in a series of chemically defined media in which the essential amino acid valine was supplied in the form of each of the Val-$(Gly)_x$ peptides.

(c) Preliminary studies on peptide uptake using β-casein-derived peptides (strain E8 only).

Information based on more limited study of three other strains (*L. cremoris* ML3, *L. cremoris* SK11 and *L. cremoris* AM2) is presented since these three strains showed significant differences in their growth responses and/or in their uptake of peptides, from those observed for *L. cremoris* E8 and *L. lactis* 920.

Because the different strains studied revealed different results with respect to growth and peptide uptake, which in turn influenced the types of experiments carried out, the results for each strain will be presented separately.

4.1 *Lactococcus lactis* 1403

As explained above, *L. lactis* 1403 was chosen for the initial work on peptide utilisation as it was the first strain obtained which showed rapid and reproducible growth in chemically defined medium (CDM).

4.1.1 Growth of *L. lactis* 1403 in media containing valine supplied in different forms.

The growth of this strain was investigated on seven media which differed in their nutritional complexity and particularly in the nature of the valine source. The media used were two complex media, skim milk medium (SMM) and a casein hydrolysate medium (CHM), and a range of chemically defined media (CDM) in which the essential amino acid valine was supplied in different forms. These forms ranged from free valine to the tetrapeptide Val-Gly_3, or to protein-bound valine in the β-casein defined medium (β-CDM). The composition of these media is described in detail in Section 2.2.1.

The maximum growth rates of cells in CDM supplemented with different sources of valine, and in CHM, are very similar (Table 4.1.1.1),and all demonstrate sigmoidal-type growth (Figure 4.1.1.1). It is of interest to note that the growth rate on CDM containing valine solely in the form of the tetrapeptide, Val-$(Gly)_3$, is not significantly less than that in media containing valine as the free amino acid, or as a dipeptide or tripeptide. This result suggests that, at the peptide concentration used, *L. lactis* 1403 cells can readily utilise a tetrapeptide as the sole source of an essential amino acid. (In subsequent work with *L. cremoris* E8, the question as to whether this would also be true at a growth limiting concentration of peptide is addressed). Cells grown in SMM and β-CDM showed biphasic-type growth (Figure 4.1.1.1) which probably reflects the more complex form in which essential amino acids were supplied.

Table 4.1.1.1 Specific growth rates of *L.lactis* subsp. *lactis* 1403 on different media

Media	Maximum Growth Rate(s) [a]
Skim Milk Medium (SMM)	0.856 and 0.128[b]
Casein Hydrolysate Medium (CHM)	0.770
β-casein Defined Medium (β-CDM)	0.334 and 0.122[b]
Chemically Defined Medium (CDM)	0.630
CDM + Val-Gly	0.693
CDM + $Val\text{-}Gly_2$	0.770
CDM + $Val\text{-}Gly_3$	0.770

[a] determined over the logarithmic phase of growth using the expression $\frac{\log_e 2}{\text{doubling time}}$.

[b] the two values given for growth rates on SMM and β-CDM represent the rates for the initial rapid and subsequent slow phases of growth (see Figure 4.1.1.1).

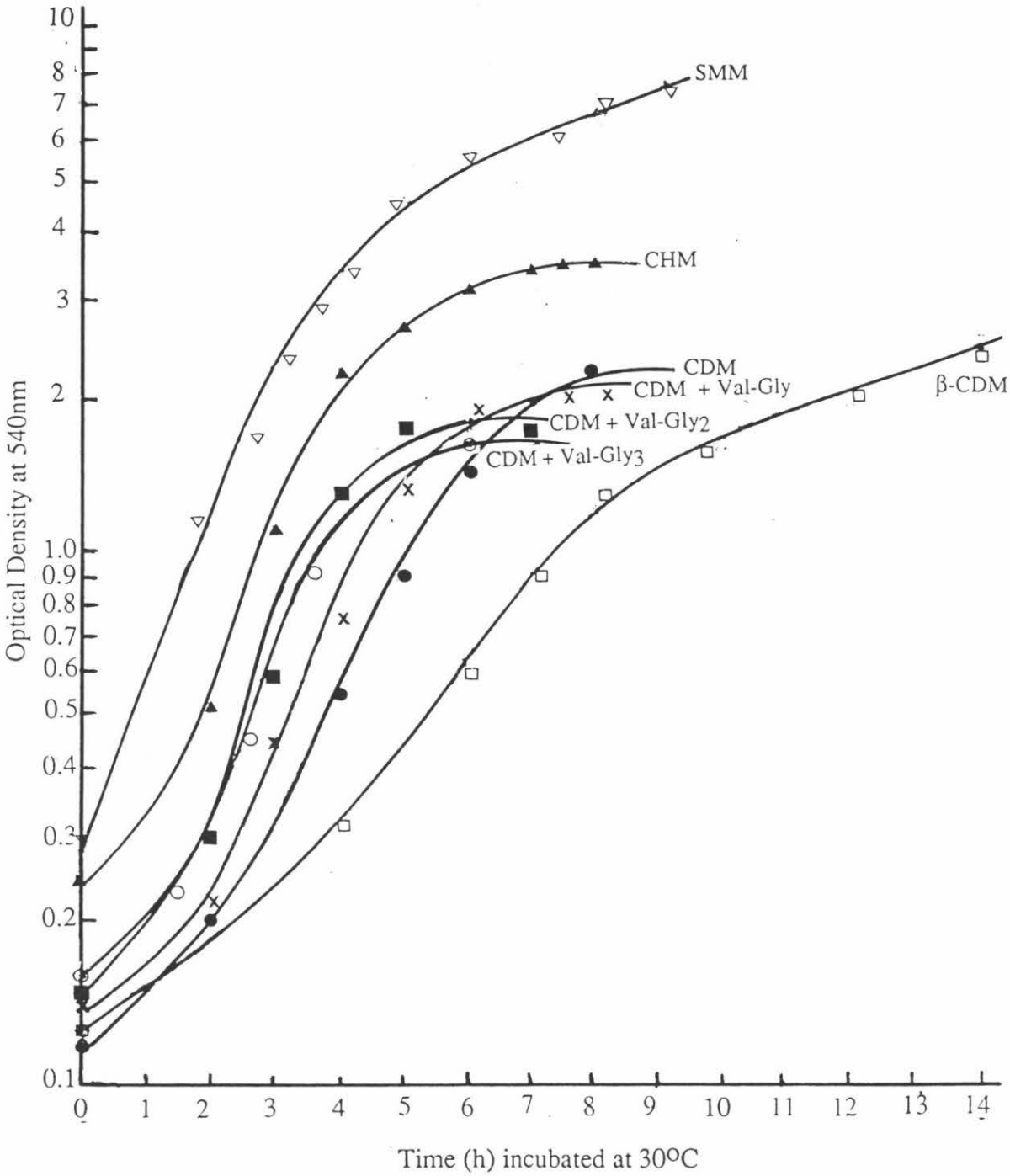


Figure 4.1.1.1 Growth of *L.lactis* subsp *lactis* 1403 in media containing1.5 % (w/v) lactose, and different nitrogen sources.

Chemically defined medium (CDM) containing 3.6 mM Val	(●)
CDM in which free valine is replaced by 3.6 mM Val-Gly	(X)
CDM in which free valine is replaced by 3.6 mM Val-Gly2	(■)
CDM in which free valine is replaced by 3.6 mM Val-Gly3	(○)
CDM (β-CDM) in which the sole amino acid source is 1 % β-casein	(□)
Casein Hydrolysate Medium (CHM)	(▲)
Skim Milk Medium (SMM)	(▽)

Cells were grown through two passages of the medium of interest before measurements were taken.

Higher growth yields were generally seen in media with complex nitrogen sources (SMM and CHM) than in chemically defined media.

4.1.2 Peptidase activities of *L. lactis* 1403 grown on different nitrogen forms.

The activities of six different peptidases were determined in French Press extracts of cells harvested from six of the seven growth runs described in the previous section (Table 4.1.2.1).
Peptidase activities were not determined in cells grown in β-CDM since cultures were grown on a small scale which yielded insufficient quantities of cells for enzyme activity measurements.
Dipeptidase, tripeptidase, proline iminopeptidase and X-prolyl dipeptidyl aminopeptidase activities were similar in cells grown in different media. Lysine aminopeptidase activities were also similar except in cells grown in CDM + Val where activity was considerably higher and in CDM + Val-Gly were activity was low compared with that in other media. Prolidase activities showed the most variation and it is difficult to identify any clear pattern.
In general, there is no clear evidence for significant induction of particular peptidases in any of the media.

4.1.3 Uptake of Val-$(Gly)_x$ peptides by *L. lactis* 1403

The rate of uptake of Val-Gly, Val-Gly_2 and Val-Gly_3 by washed cell suspensions of *L. lactis* 1403, grown on CDM, was followed by taking samples at regular time intervals and measuring the rate of decrease in peptide concentration in the supernatant by capillary electrophoresis (CE). After two hours, the cells were removed by centrifugation and subsequent filtration, and a further aliquot of peptide was added to the supernatant. Timed samples were taken over another 60 min to assess whether extracellular peptidase activity was present. The details of this procedure are described in Section 2.2.10.

The capillary electrophoresis profiles obtained during measurement of the uptake of Val-Gly_2 by strain 1403 are shown in Figure 4.1.3.1. Conversion of the CE peak areas to peptide concentrations is detailed in Table 4.1.3.1. A graph plotting the rate of peptide removal from the cell suspension supernatant, and subsequently in the cell-free supernatant, is shown in Figure 4.1.3.2. Calculations used to determine the net rate of peptide uptake from this data are presented in Figure 4.1.3.3.

Table 4.1.2.1 Peptidase activities of *L.lactis* 1403 cells grown on different nitrogen sources. Peptidase activities are expressed as μmol min^{-1} mg^{-1}protein

Media	Dipeptidase Ala-Ala[a]	Tripeptidase Leu-Gly_2	Prolidase Leu-Pro	Pro/imino-peptidase Pro-Ala	Lys-amino peptidase Lys-*p*NA	X-prolyl dipeptidyl aminopeptidas Gly-Pro-AMC
SMM	2.99	0.421	0.230	0.018	0.075	0.157
CHM	3.14	0.418	0.093	0.026	0.047	0.223
CDM	3.99	0.432	0.321	0.016	0.124	0.228
CDM+ Val-Gly	2.21	0.301	0.087	0.009	0.015	0.240
CDM+ Val-Gly_2	3.58	0.481	0.289	0.016	0.031	0.206
CDM+ Val-Gly_3	4.60	0.674	0.136	0.012	0.065	0.265

[a] substrate used for assay of peptidase activities.

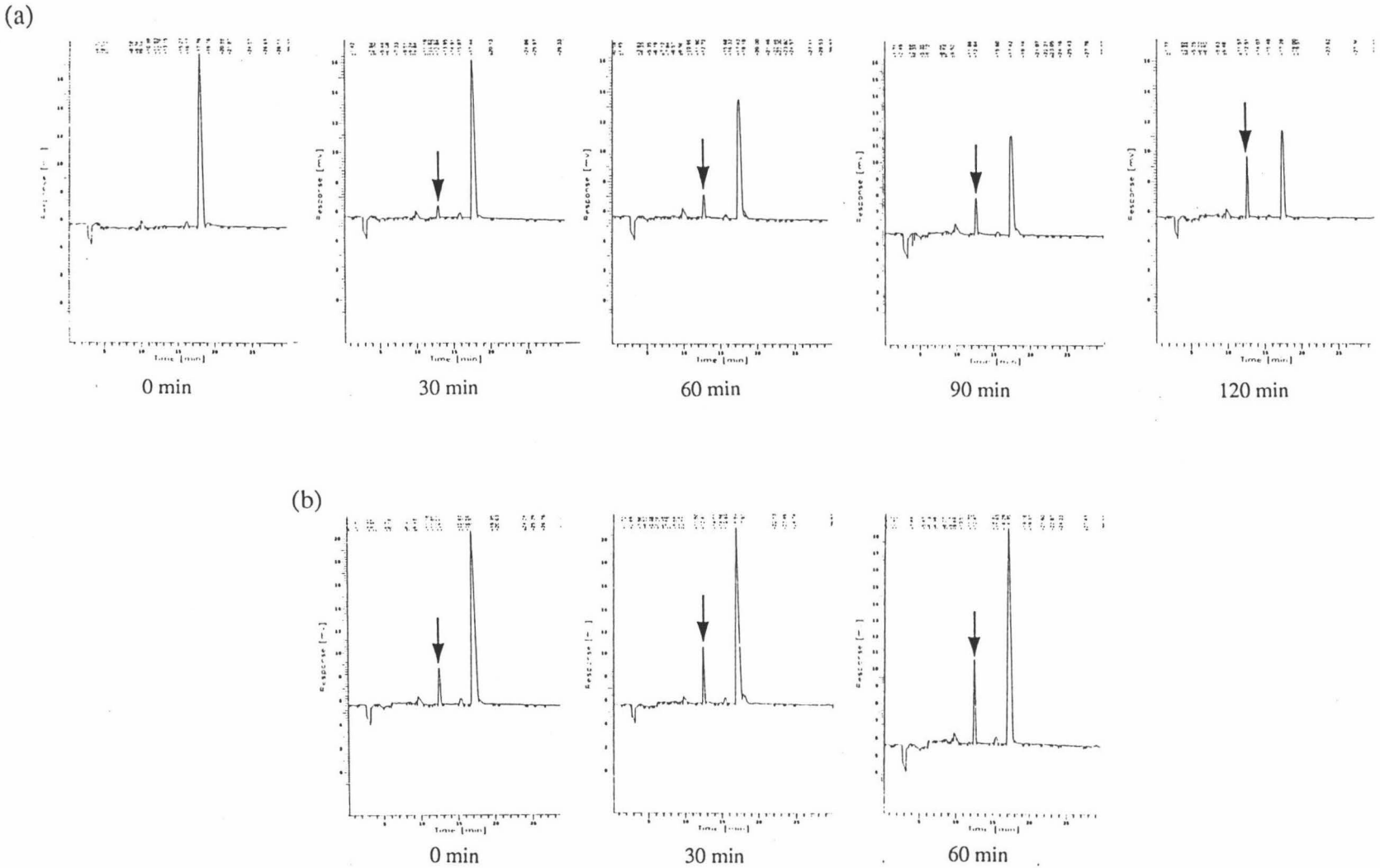


Figure 4.1.3.1 Capillary electrophoresis profiles from a time course study of Val-Gly_2 uptake by glycolysing cell suspensions of *L. lactis* subsp. *lactis* 1403. (a) Val-Gly_2 removal from the supernatant in the presence of whole cells of *L. lactis* 1403. (b) Val-Gly_2 removal from the cell-free supernatant by extracellular peptidases. The arrow identifies the appearance of a peak that corresponds to the dipeptide Gly-Gly. A 2.5 sec injection was made onto a 72 cm Freezone column. Peptides were eluted by running in 20 mM sodium citrate pH 2.5 at 30 kV for 30 min.

Table 4.1.3.1 Conversion of the capillary electrophoresis area data for Val-Gly_2 utilisation into actual concentrations. (a) Val-Gly_2 removal from the supernatant of washed cell suspensions of *L.lactis* subsp *lactis* 1403 (b) Val-Gly_2 removal by peptidases in the cell-free supernatant.

(a)

Time of Sample (min)	Area of CE Peak	Area as a % of 0 min Sample	Val-Gly_2 Concentration (mM)
0	359089.5	100	3.82
30	303651.0	84.6	3.23
60	251824.5	70.1	2.68
90	180432.5	50.2	1.91
120	143050.5	39.8	1.52

(b)

Time of Sample (min)	Area of CE Peak	Area as a % of 0 min Sample	Val-Gly_2 Concentration (mM)
0	455471.5	100	4.85
30	397955.2	87.4	4.23
60	359447.0	78.9	3.83

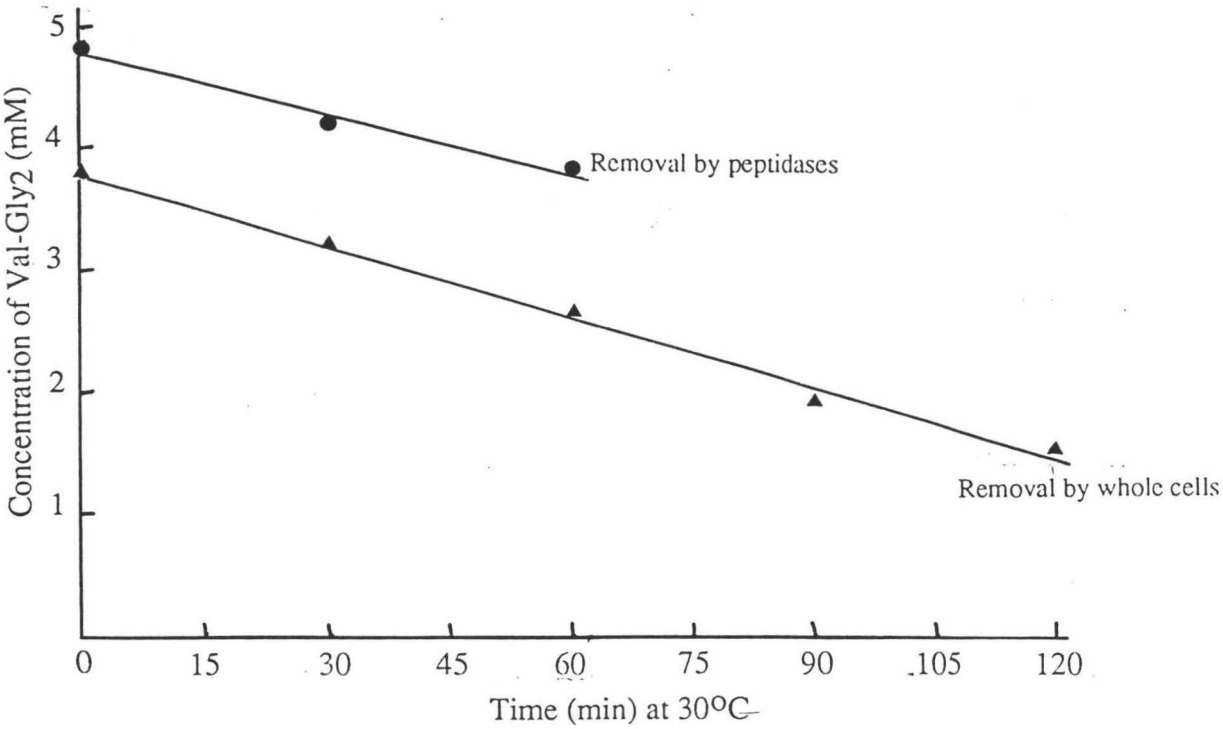


Figure 4.1.3.2 Graph showing the decrease in Val-Gly$_2$ concentration when this peptide was incubated with cell suspensions of *L.lactis* 1403, and in a subsequent incubation with the cell-free supernatant. Refer to text for a detailed explanation.

(A) The rate of peptide removal by whole cells = $\frac{3.78 - 1.4}{120\text{min}} \times 60\text{min}$

$= 1.19\ \text{mM.h}^{-1}$

$= (1.19 \times 1.5/1000 \times 10^6)\ \text{nmol.h}^{-1}$

$= 1785\ \text{nmol.h}^{-1}.0.710\text{mg}^{-1}\text{dwc}^*$

$= 2514\ \text{nmol.h}^{-1}.\text{mg}^{-1}\text{dwc}$

(B) The rate of peptide removal due to peptidases = $\frac{4.80-3.76}{60\text{min}} \times 60\text{min}$

$= 1.04\ \text{mM.h}^{-1}$

$= (1.04 \times 1.5/1000 \times 10^6)\ \text{nmol.h}^{-1}$

$= 1560\ \text{nmol.h}^{-1}.0.710\text{mg}^{-1}\text{dwc}$

$= 2197.18\ \text{nmol.h}^{-1}.\text{mg}^{-1}\text{dwc}$

Net rate of peptide uptake by whole cells = (A) - (B)

$= (2514.08 - 2197.18)\ \text{nmol.h}^{-1}.\text{mg}^{-1}\text{dwc}$

$= 316.9\ \text{nmol.h}^{-1}.\text{mg}^{-1}\text{dwc}$

Notes: * dwc = dry weight cells.

Cell density was determined by comparing the optical density at 540nm of a suitably diluted sample of the cell suspension with a standard curve of OD_{540nm} versus cell dry weight.

Figure 4.1.3.3 Summary of the procedure used for calculating the net rate of peptide uptake by whole cells.

A summary of the results obtained for the net rates of uptake of the three synthetic peptides by *L. lactis* 1403 is shown in Table 4.1.3.2.

Table 4.1.3.2. Net rates of uptake of Val-$(Gly)_x$ peptides by *L. lactis* subsp *lactis* 1403

Peptide	Peptide Removal Rate by cell suspensions (nmol h^{-1} mg^{-1})	Peptide Removal Rate due to Peptidase Activity (nmol h^{-1} mg^{-1})	Net Rate of Peptide Uptake by Cells (nmol h^{-1} mg^{-1})
Val-Gly	1418.4	1598.6	0
Val-Gly_2	2514.08	2197.18	317
Val-Gly_3	1353.7	204.9	1149

L. lactis 1403 cells grown in CDM were resuspended in 6.5 ml of incubation buffer. After incubating at 30 °C for 10 min to de-energise, glucose was added to a final concentration of 0.2 % (w/v). After a further 15 min Val-Gly, Val-Gly_2 or Val-Gly_3 was added to final concentration of 3.8 mM. Timed samples were then taken at regular intervals over the next 120 min. At the end of this period the cells were removed by centrifuging at 7000g for 5 min at 4 °C. The supernatant was filtered through a 0.22 µM Millipore filter and a further aliquot of peptide was added. Timed samples were then taken over a further 60 min to assess extracellular peptidase activity.

The results from these experiments demonstrate the importance of carrying out controls for peptidase activity in the medium. In this procedure the removal of a substrate from the supernatant around whole cells is being measured, rather than following the accumulation of that substrate inside whole cells . (In lactococci measurement of peptide accumulation is not possible as peptides are rapidly hydrolysed into their constitutive amino acids upon entry into the cell). The possibility therefore exists that the decline in supernatant peptide levels observed may in fact be due to peptidase activity in the medium rather than transport of the intact peptide.

This is clearly the situation with *L. lactis* 1403. In the case of Val-Gly , it was found that the rate at which this peptide was cleaved by dipeptidase was greater than the rate of disappearance seen with the whole cell suspension. It therefore appears that no peptide was transported intact.

Similarly, removal of the tripeptide Val-Gly_2 from the incubation buffer by *L. lactis* 1403 can almost entirely be attributed to peptidase cleavage. (Although a small net rate of uptake is seen [Figure 4.1.3.3 and Table 4.1.3.2], because of the relatively large error in determining accurate rates from a relatively small number of time points, little significance can be attributed to this rate. In later experiments with other strains, more frequent time points were taken to improve the accuracy of rate determinations). The conclusion that removal of Val-Gly_2 from the incubation buffer is due principally to cleavage by tripeptidase activity is supported by the CE data which shows the appearance of a second major peak over the incubation period (Figure 4.1.3.1). This peak was identified by running a standard to be the dipeptide Gly-Gly (data not shown). This is the expected product of Val-Gly_2 hydrolysis by tripeptidase action. The di-L-glycine product of this cleavage is known to be resistant to hydrolysis by the lactococcal dipeptidase (Hwang *et al.*, 1981).

This ability of CE to resolve closely related peptides therefore provides an important tool for assessing whether a peptide is being hydrolysed or not. Hydrolysis of any potential transport substrate can be tested for simply by looking for the appearance of potential cleavage products.

The absence of any products of Val-Gly_3 cleavage by peptidase activity is consistent with the small value calculated for peptide removal by the supernatant once cells have been removed.(Table 4.1.3.2) This peptide would not be a substrate for the lactococcal dipeptidase or tripeptidase. It could however be hydrolysed by the "lysyl" aminopeptidase which is known to have a relatively broad specificity. Although the activity of individual peptidases in the supernatant was not measured, it is most likely that the supernatant peptidase activities arose from autolysis of a portion of the *L. lactis* 1403 cells.(see Section 4.1.4 below). It is therefore reasonable to assume that the relative activity of the peptidases in the supernatants might reflect that of the intracellular peptidases. Earlier results which determined the various activities of the intracellular peptidase complement of *L. lactis* 1403 on different media, had shown lysine aminopeptidase activity to be significantly less than that of the dipeptidase and tripeptidase (Table 4.1.2.1), particularly as valyl substrates have been shown to be hydrolysed at about 1 to 2 % of the rate of lysyl substrates by lactococcal aminopeptidase (Midwinter, 1992).
These considerations support the conclusion that the net rate of Val-Gly_3 removal in cell suspensions of *L. lactis* 1403 is a valid measure of uptake by whole cells

and indicate that this oligopeptide can be transported at a significant rate by this strain.

4.1.4 Assessment of leakage of intracellular enzymes from *L. lactis* 1403 cells

The results presented above support the general conclusion drawn by Law (1977) that *L. lactis* subsp. *lactis* strains, in contrast to *L. .lactis* subsp. *cremoris* strains, hydrolyse dipeptides extracellularly prior to their transport as free amino acids. Law (1977) suggested that hydrolysis was may be due to the activity of a dipeptidase secreted specifically. It is equally as possible that this extracellular peptidase activity is the result of autolysis which leads to the non-specific release of enzymes. In order to test this possibility, levels of the intracellularly located enzyme, aldolase, were measured in the supernatant obtained by incubating *L. lactis* 1403 cells under the same conditions used as those in the uptake experiments (Table 4.1.4.1).

Table 4.1.4.1 Assessment of intracellular adolase leakage from incubating whole cells of *L. lactis* subsp *lactis* 1403. Cells were grown and incubated using the same procedure and conditions as those used in the peptide uptake studies, but in the absence of peptide.

Time (min)	Aldolase Activity (μmol min^{-1} ml^{-1})		
	(A) Supernatant	(B) French Press Extract	A/B x 100 (%)
0	0	0.195	0
30	3.2×10^{-2}	0.184	17.4
60	5×10^{-2}	0.130	38.46

(Aldolase levels were determined using the method of Crow *et al.*, 1982)

These results showed that there was significant leakage of aldolase from *L. lactis* 1403 cells. This strongly suggests that the extracellular hydrolysis of Val-Gly and Val-Gly_2 seen in the whole cell uptake experiments is the result of leaked intracellular peptidases.

The finding that hydrolysis of peptides by leaked internal peptidases contributes to a large extent to the measured rate of peptide removal, seriously detracts from the procedure used in this study to measure rates of peptide uptake by *L. lactis* 1403 as the leaked peptidases may be hydrolysing peptides that would normally be transported intact. In an attempt to assess whether cells of this strain could transport di- and tripeptides without hydrolysis, the peptides D-Leu-Gly and D-Leu-Gly_2 were used as substrates for the peptide uptake system. These peptides were found not to be hydrolysed by the intracellular peptidases of *L. lactis* 1403 (data not shown). An uptake experiment using the procedure described previously revealed no removal of either peptide by cell suspensions. Presumably these peptides cannot be recognised as substrates by the di-/tripeptide carrier system.

In light of this significant cell leakage it was decided to abandon further work on *L. lactis* 1403. Studies of peptide utilisation by another *L. lactis* strain, 920, are described in a later section.

4.2 *Lactococcus cremoris* E8

The *L. lactis subsp. cremoris* E8 was selected as a typical representative of the *cremoris* group of lactococci. This strain has been widely used in a number of laboratories for both fundamental and applied investigations on lactococci, including some previous work on peptide transport (Law, 1977; 1978). This work of Law indicated that dipeptides are transported into *L. cremoris* E8 cells without prior hydrolysis.
It is with this strain that we have carried out our most extensive research into lactococcal peptide utilisation. A number of preliminary studies were carried out to assess its suitability for investigation of peptide transport under the conditions used in this research. Information is then presented from uptake and growth studies with the model Val-$(Gly)_X$ peptide series, and from preliminary work on the uptake of β-casein peptides.

4.2.1 Growth and peptidase activities of *L. cremoris* E8 in chemically defined medium.

Due to time constraints , it was not feasible to repeat with *L. cremoris* E8 the extensive work carried out with *L. lactis* 1403 to assess the growth of this strain upon various media (Section 4.1.1), and to assess whether the internal peptidase complement of these cells was nutritionally regulated (Section 4.1.2). However as part of the emphasis in this work on the possible differences of individual lactococcal strains, it was important to compare growth and peptidase activities of *L. cremoris* E8 and *L. lactis* 1403 cells grown in chemically

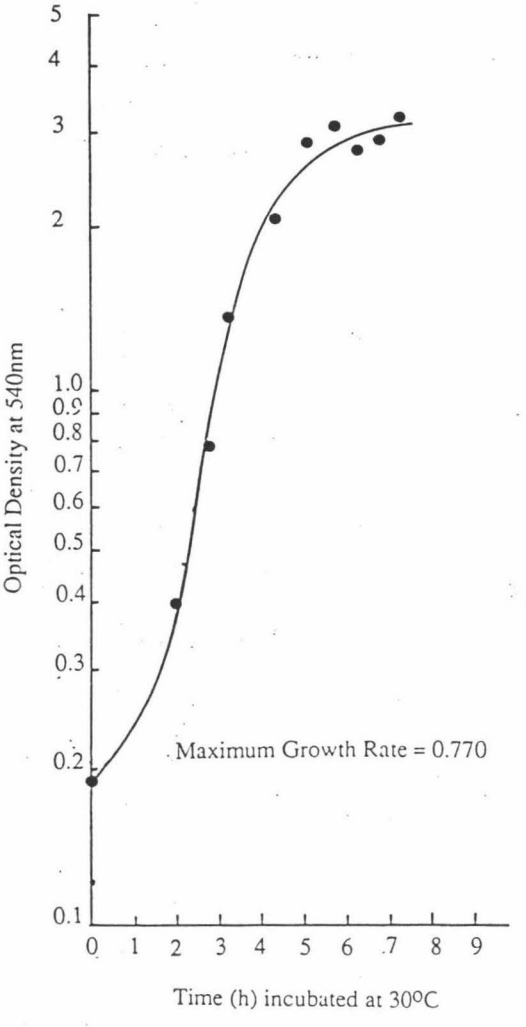


Figure 4.2.1.1 Growth of *L.lactis* subsp *cremoris* E8 in chemically defined medium, in which all amino acids are supplied in their free state, and containing 1.5% (w/v) lactose. Cells were grown through two passages of this medium before measuremenrts were taken.

Table 4.2.1.1 Peptidase activities of a French Press extract of *L. cremoris* E8 cells grown in chemically defined medium. Peptidase activities are expressed as μmol. $min^{-1}.mg^{-1}$ protein.

Strain	Dipeptidase Ala-Ala[a]	Tripeptidase Leu-Gly_2	Prolidase Leu-Pro	Pro/imino-peptidase Pro-Ala	Lys-amino peptidase Lys-*p*NA	X-prolyl dipeptidyl aminopeptidase Gly-Pro-AMC
E8	1.71	0.483	0.128	0.008	0.126	0.261

[a] substrate used for the assay of peptidase activities.

defined media. *L. cremoris* E8 grew well in CDM with a maximum growth rate of 0.770, reaching a maximum OD_{540nm} of 3.1 (Figure 4.2.1.1). Both parameters are higher than what was found with *L. lactis* 1403 in the same medium. The activities of intracellular peptidases determined in cell-free extracts of *L. cremoris* E8, were mostly similar to those of *L. lactis* 1403 (Table 4.2.1.1), although the dipeptidase, prolidase and proiminopeptidase activities are all somewhat lower than those of the 1403 strain.

4.2.2 Assessment of intracellular leakage from *L. cremoris* E8 cells.

As discussed in Section 4.1.4, the finding that a significant amount of Val-Gly and $Val\text{-}Gly_2$ removal from the supernatant of whole cell suspensions of *L. lactis* 1403 was due to the activity of leaked intracellular peptidase was a major obstacle to attempts to measure peptide uptake in this strain.
It was therefore important to determine whether peptidases also leaked from *L. cremoris* E8 cells before proceeding with transport studies. Aldolase levels from the supernatant of incubated washed cells of *L. cremoris* E8, previously grown in CDM, are shown in Table 4.2.2.1. Supernatant aldolase levels were found to be less than one percent of the total cellular aldolase activity levels. These results suggest that *L. cremoris* E8 is not prone to significant autolysis under the experimental conditions used.

Table 4.2.2.1. Assessment of intracellular adolase leakage from whole cells of *L. lactis* subsp *cremoris* E8. Cells were incubated under the same conditions as used in the peptide uptake experiments, except for the omission of peptide.

Time (min)	Aldolase Activity ($\mu mol\ min^{-1}\ ml^{-1}$)		
	(A) Supernatant	(B) French Press Extract	A/B x 100 (%)
0	8.76×10^{-4}	0.131	0.67
30	1.46×10^{-3}	0.175	0.83
60	0	0.203	0

(Aldolase levels were determined using the method of Crow *et al.*, 1982)

4.2.3 Uptake of Val-$(Gly)_X$ peptides by *L. cremoris* E8

The rates of uptake of the synthetic peptides Val-Gly, Val-Gly_2, Val-Gly_3, Val-Gly_4, and Val-Gly_7 by washed whole cell suspensions of *L. cremoris* E8 were determined using the method described in Section 2.2.1 and the calculations detailed in Section 4.1.3. A summary of the net peptide uptake rates is presented in Table 4.2.3.1.

Capillary electrophoresis profiles for Val-Gly_2 uptake by *L. cremoris* E8 are shown in Figure 4.2.3.1(a). Examination of these profiles provides evidence that the tripeptidase activity seen in supernatants of *L. lactis* 1403 cell suspensions is not detectable in *L. cremoris* E8 supernatants.
The peak corresponding to diglycine that appeared in the *L. lactis* 1403 profiles (Figure 4.1.3.1(a)) as a result of enzymatic hydrolysis of Val-Gly_2, is absent from the corresponding profiles for strain E8.
Furthermore, once *L. cremoris* E8 cells were removed from the incubation buffer, peptide levels in the supernatant ceased to decline (Figure 4.2.3.1(b)). This was not the case with strain 1403, where peptide levels continued to decline in the cell-free supernatants because of extracellular peptidase activity (Figure 4.1.3.1(b)).
These observations support the conclusion that, in *L. cremoris* E8, the measured rate of di- and tripeptide uptake were due to their transport intact.

To conserve the very limited quantities of the synthesised peptides, Val-Gly_4 and Val-Gly_7, peptidase controls were omitted from uptake experiments with these two oligopeptides. As described above, the absence of detectable peptidase leakage from *L. cremoris* E8 cells, established in the uptake work with the smaller Val-$(Gly)_X$ peptides, made such controls unnecessary. If enzymes capable of cleaving these larger peptides were released due to some unexpected cell autolysis, then the products of hydrolysis should have been apparent in the CE profiles. The profiles for Val-Gly_7 uptake by cell suspensions of strain E8 (Figure 4.2.3.2) show only the Val-Gly_7 peptide peak This results suggests that oligopeptides are also transported into this strain intact.

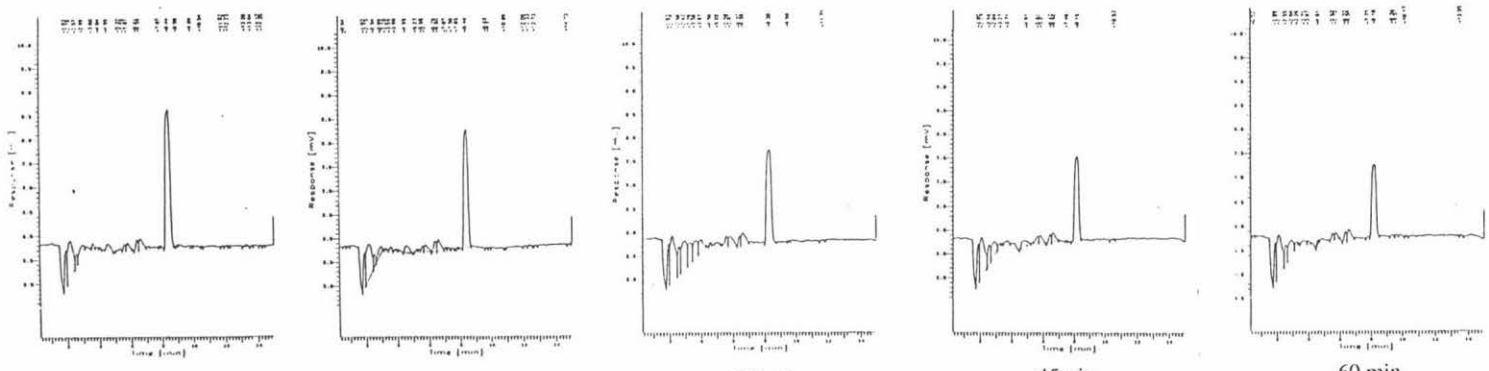
An attempt was made to assess transport of the decapeptide Val-Gly_9. However the results obtained from CE analysis of the data were unsatisfactory. It was not possible to repeat this experiment because of the difficulties in obtaining quantities of this peptide at the level of purity needed for these studies (see Section 3.1.4).

Table 4.2.3.1 Net Rates of uptake of Val-$(Gly)_x$ peptides by *L. lactis* subsp *cremoris* E8

Peptide	Peptide Removal Rate by Cell Suspensions (nmol h^{-1} mg^{-1})	Peptide Removal Rate due to Peptidase Activity (nmol h^{-1} mg^{-1})	Net Rate of Uptake by Cells (nmol h^{-1} mg^{-1})
Val-Gly	760.86	0	761
Val-Gly_2	871.90	0	872
Val-Gly_3	731.7	0	731
Val-Gly_4	866.1	ND	866
Val-Gly_7	777.0	ND	777

L. cremoris E8 cells grown in CDM were resuspended in 6.5 ml of incubation buffer. After incubating at 30 °C for 10 min to de-energise, glucose was added to a final concentration of 0.2 % (w/v). After a further 15 min Val-Gly, Val-Gly2, Val-Gly_3, Val-Gly_4 or Val-Gly_7 was added to final concentration of 1 mM. Timed samples were then taken at regular intervals over the next 120 min. At the end of this period the cells were removed by centrifuging at 7000g for 5 min at 4 °C. The supernatant was filtered through a 0.22 μM Millipore filter and a further aliquot of peptide was added. Timed samples were then taken over a further 60 min to assess extracellular peptidase activity.

(a)



(b)

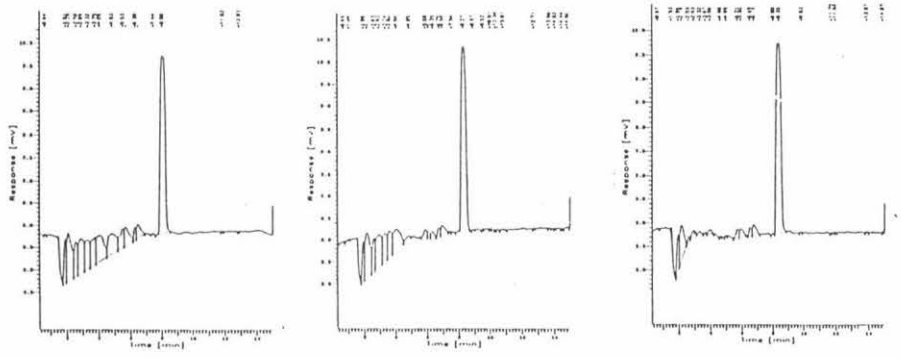


Figure 4.2.3.1 Capillary electrophoresis profiles from a time course study of Val-Gly_2 uptake by glycolysing cell suspensions of *L.lactis* subsp. *cremoris* E_8. (a) Val-Gly_2 removal from the supernatant in the presence of whole cells of *L.cremoris* E_8. (b) Assessment of Val-Gly_2 removal from the cell-free supernatant by extracellular peptidases. A 2.5 sec injection was made onto a 72 cm Freezone column. Peptides were eluted by running in 20 mM sodium citrate pH 2.5 at 30 kV for 30 min.

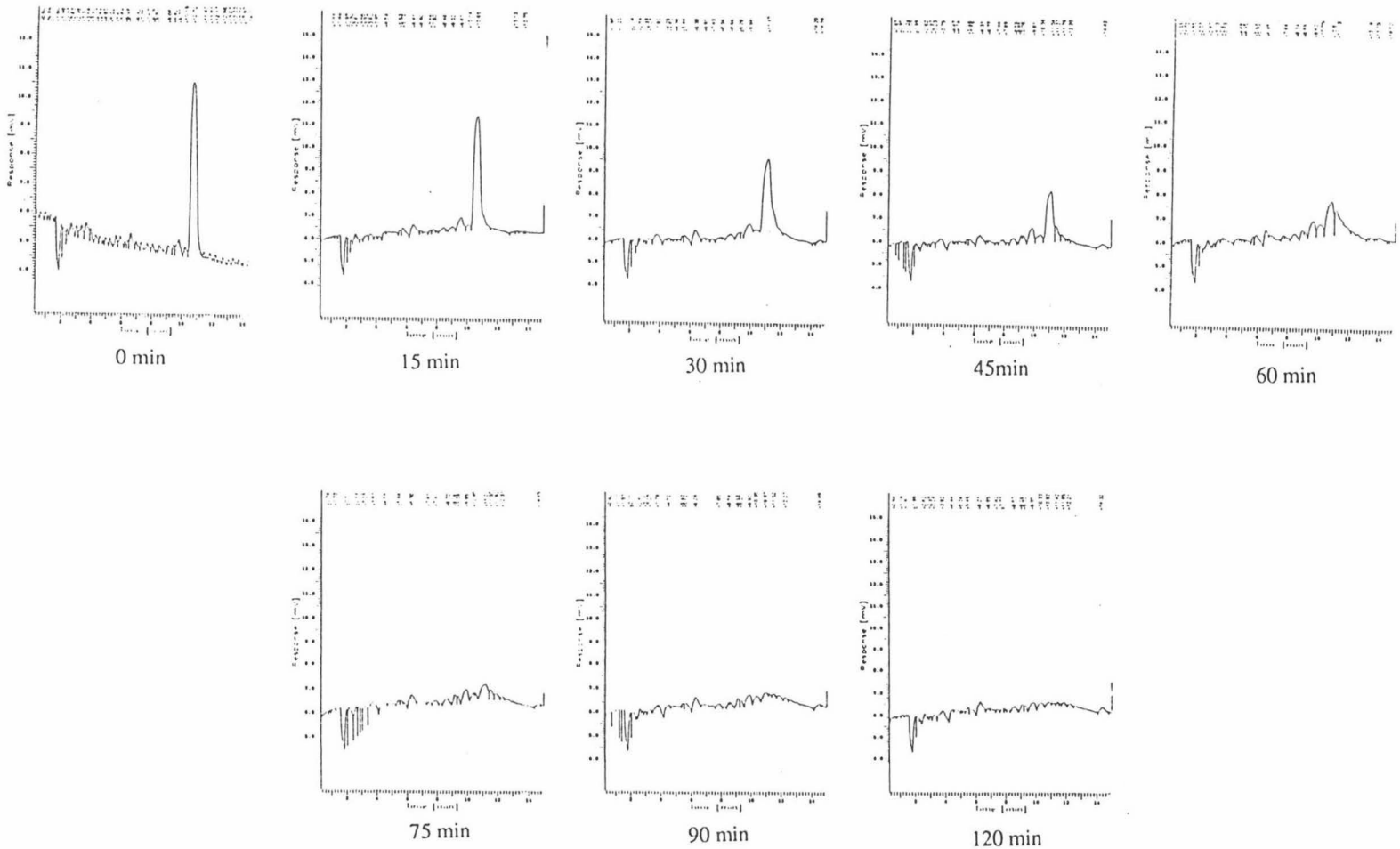


Figure 4.2.3.2 Capillary electrophoresis profiles from a 120 min time course study of Val-Gly7 uptake by glycolysing cell suspensions of *L.lactis* subsp. *cremoris* E8.

A 2.5 sec injection was made onto a 72 cm Freezone column. Peptides were eluted by running in 20 mM sodium citrate pH 2.5 at 30 kV for 30 min.

The results presented on the relative rates of uptake of the different Val-Gly_X peptides are significant for two reasons. Firstly previous investigations, using the *L. cremoris* strain ML_3, into the relative activities of the di-/tripeptide transport system and the oligopeptide transport system, suggested that the oligopeptide transport system is much less active than the di-/tripeptide transport system (Smid, 1991; Kunji *et al.*, 1993). The results of the present study indicate that oligopeptide uptake rates by whole cells of *L. cremoris* E_8 are as high as the rates of uptake of the corresponding di- and tripeptides. Secondly, these results support recent studies (Tynkkynen *et al.*, 1993) which demonstrate that peptides containing up to 8 residues are capable of being transported by the oligopeptide transport system in lactococci. This is larger than a previously widely accepted upper limit of 5 residues based on earlier studies (Law, 1978; Rice *et al.*, 1978).

4.2.4 Utilisation of Val-$(Gly)_X$ peptides as a source of valine for growth of *L. cremoris* E_8 in chemically defined media.

Growth studies in chemically defined media are useful for testing the effect of a single parameter, such as a peptide, upon cell growth. In addition to determining the rate of uptake of Val-$(Gly)_X$ peptides by cell suspensions, the utilisation of these peptides was also investigated by determining the ability of cultures to use them for growth in a chemically defined medium.

A major reason for selecting valine as the basis of the synthetic peptide series was that it is essential for the growth of both *L. lactis* and *L. cremoris* strains. The results from earlier growth work with *L. lactis* 1403 showed that this strain was able to utilise a range of peptides as sources of this essential amino acid (Section 4.1.1). However, the concentration of peptides used in the growth experiments with *L. lactis* 1403 were relatively high. In order to draw quantitative conclusions concerning the relative activities of the different peptide transport systems, growth measurements need to be carried out at rate-limiting concentrations of the valine source.

For example, if the oligopeptide system in strain E_8 is as active as the di-/ tripeptide transport system as suggested by the results of the uptake experiments (Table 4.2.3.1), then similar growth rates should be seen in media supplying valine in the form of di- or tripeptides, or in the form of valine oligopeptides such as Val-Gly_3, Val-Gly_4 or Val-Gly_7. In contrast, if significant differences exist between the activity of these two transport systems, as suggested from earlier work, then the rate of growth of strain E_8 in media where Val-Gly or Val-Gly_2 are sole sources of valine, should be much faster than in media where valine is supplied as Val-Gly_3, Val-Gly_4 or Val-Gly_7.

In order to determine an appropriate growth-limiting concentration of valine source for such experiments, growth rates of *L. cremoris* E8 were determined in CDM over a range of different valine concentrations (Table 4.2.4.1).

Table 4.2.4.1 Determination of the growth rates of *L. lactis* subsp. *cremoris* E8 on chemically defined media supplemented with different concentrations of the essential amino acid valine.

Concentration of valine in the CDM (mg l^{-1})	Concentration of valine in the CDM (mM)	Growth Rate.
400[a]	3.42	0.693
200	1.71	0.693
100	0.85	0.198
50	0.42	0.066
25	0.21	0
0	0	0

[a] This is the concentration of valine used in the growth experiments carried out previously with *L.lactis* 1403

L.cremoris E8 cells were grown through two generations of the media of interest before growth measurements were taken.

Reduction of the valine concentration to 0.85 mM resulted in marked reduction in growth rates of the culture. It was not feasible to carry out this determination with the limited quantities of synthetic Val-$(Gly)_X$ peptides available so it was assumed that this concentration would also be growth rate limiting when the valine was supplied as a peptide form.

Chemically defined media were constructed containing Val-$(Gly)_X$ peptides at this concentration. Samples of each medium were subjected to acid hydrolysis and amino acid analysis to check that the levels of available valine in each were comparable (Table 4.2.4.2).

Table 4.2.4.2 Analytically determined valine concentrations in the various chemically defined media used in growth experiments with *L.cremoris* E8.

Media	nmol Val $100\mu l^{-1}$ of Media
CDM - Val	0
CDM + 0.9 mM Val	85.0
CDM + 0.9 mM Val-Gly	91.8
CDM + 0.9 mM Val-Gly_2	73.2
CDM + 0.9 mM Val-Gly_3	64.2
CDM + 0.9 mM Val-Gly_4	96.9
CDM + 0.9 mM Val-Gly_7	72.1

The growth curves obtained when *L. cremoris* E8 was grown on these media are shown in Figure 4.2.4.1. Strong growth was seen in all media where valine was supplied in peptide form, confirming that *L. cremoris* E8 is able to utilise peptides as large as 8 residues as sources of essential amino acids.
However it is also evident from the growth curves that the maximum growth rates on media containing valine in peptide form are much higher than in the medium containing valine as the free amino acid. Therefore the assumption made, namely, that the growth-rate limiting concentration of free valine would also be growth-rate limiting in the case of valine peptides, is incorrect, *i.e.*, that 0.85 mM is not the growth rate limiting concentration when valine is supplied as a Val-$(Gly)_x$ peptide.
The transport system for free valine in lactococci is also responsible for transport of leucine and isoleucine (Driessen *et al.*, 1987). Consequently, the growth limiting concentration of valine determined in the chemically defined medium

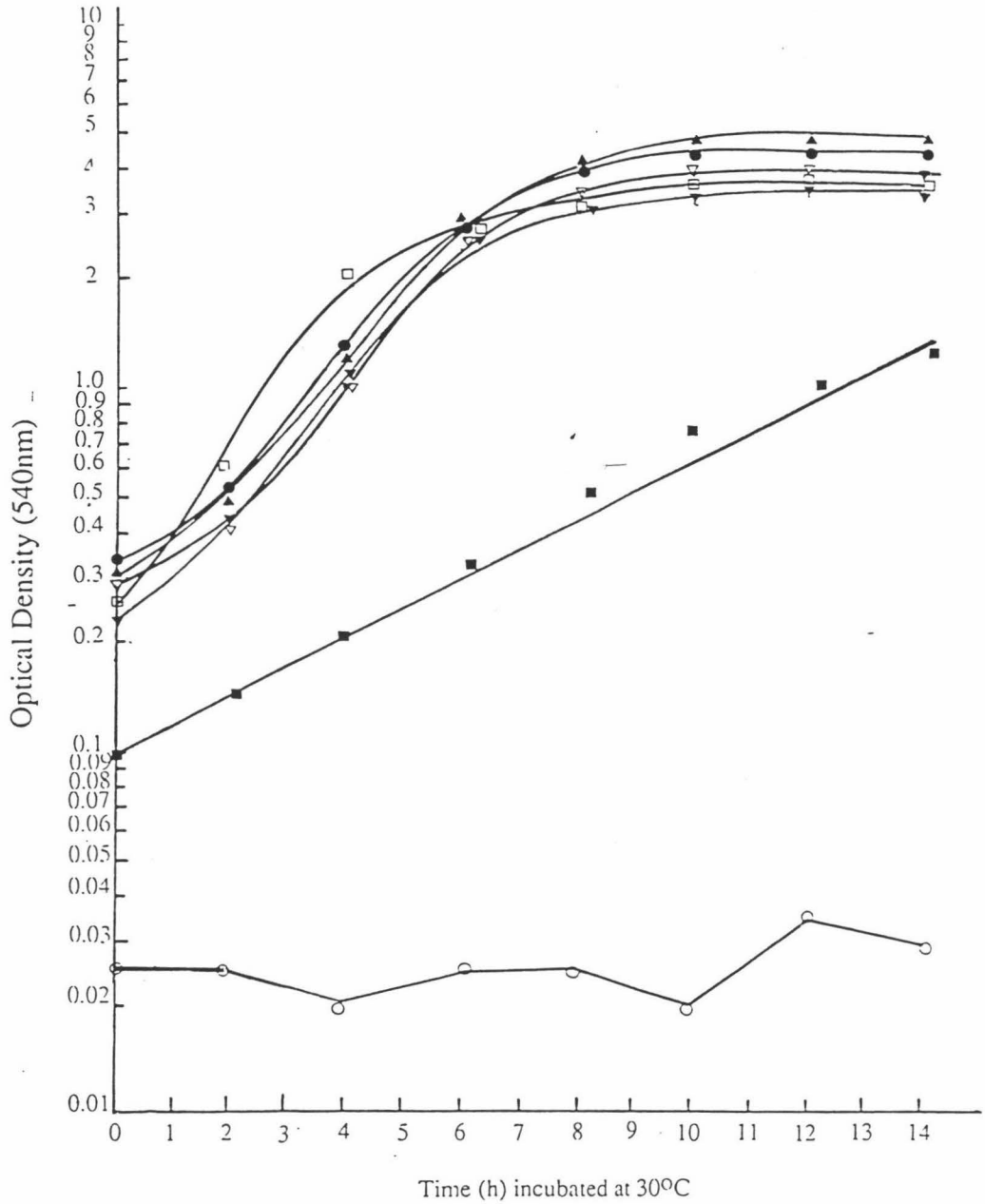


Figure 4..2.4.1 Growth of *L.lactis* subsp *cremoris* E8 in chemically defined media supplemented with 1.5 % (w/v) lactose, and different valine sources.

Chemically defined medium (CDM) without a valine source	(○)
CDM containing 0.85 mM valine	(■)
CDM in which free valine is replaced by 0.85 mM Val-Gly	(▼)
CDM in which free valine is replaced by 0.85 mM Val-Gly$_2$	(▲)
CDM in which free valine is replaced by 0.85 mM Val-Gly$_3$	(□)
CDM in which free valine is replaced by 0.85 mM Val-Gly$_4$	(▽)
CDM in which free valine is replaced by 0.85 mM Val-Gly$_7$	(●)

Cells were grown through two passages of the medium of interest before measurements were taken.

used in this study, will be that prevailing in competition with the much higher levels of isoleucine and leucine present in this medium. This competition does not exist when valine is supplied as one of the Val-$(Gly)_X$ peptides, so the limiting concentration could be much lower. Furthermore, the growth-rate limiting concentration of the different valine sources will also be dependent on the relative K_m values of the transport systems for their respective substrates. The affinities of the peptide transport systems for their substrates may be considerably higher than for the free amino acid.

However the ability of *L. cremoris* E8 to grow rapidly on all Val-$(Gly)_X$ peptides tested supports the conclusion made from the uptake studies that these peptides are being transported without prior hydrolysis. If the peptides were being cleaved as a result of dipeptidase, tripeptidase, or aminopeptidase activity, to release valine as an amino acid, then the growth rates of the cultures on the valine peptide-containing media could not be greater than that on a medium containing an equivalent concentration of free valine.
Although it is theoretically possible that a leaked endopeptidase could hydrolyse the Val-$(Gly)_X$ oligopeptides to produce Val-Gly or Val-Gly_2, this is highly unlikely from the specificity properties of the only lactococcal endopeptidase characterised to date (Pritchard *et al.*, 1994).

4.2.5 Energy dependence of Val-Gly_2 utilisation by *L. cremoris* E8

The energy dependence of Val-Gly_2 utilisation by *L. cremoris* E8 was investigated to provide further evidence that the removal of tripeptides from the supernatant of washed cell suspensions was due to a process of active transport into the cells by the di-/tripeptide transport system. Energy dependence was studied by ascertaining the effect of omission of the energy source glucose, since lactococci do not contain any endogenous reserves of carbohydrate. The effect of the ionophore carbonyl cyanide tri-chlorophenyl hydrazone (CCCP) on Val-Gly_2 uptake was also investigated since this transport system is known to be driven by a proton motive force (Smid *et al.*, 1989a).
The results obtained when Val-Gly_2 uptake by *L.cremoris* E8 cells was determined in the presence or absence of glucose, or in the presence of glucose and CCCP, are shown in Table 4.2.5.1.

Table 4.2.5.1 Demonstration of the energy dependence of Val-Gly_2 uptake by *L. lactis* subsp. *cremoris* E8.

Treatment	Val-Gly_2 Removal Rate by Cell Suspensions ($nmol.h^{-1}.mg^{-1}$)	Val-Gly_2 Removal due to Peptidase Activity ($nmol.h^{-1}.mg^{-1}$)	Net Rate of Val-Gly_2 Uptake by Cells ($nmol.h^{-1}.mg^{-1}$)
+ glucose	871.9	0	871.9
- glucose (0-30 min) (30-60 min)	 0 1312.4	 0 1809	 0 0
+ glucose (0-30 min) no CCCP (30-60 min) + CCCP	 886 0	 0 0	 886 0

L.cremoris E8 cells grown in CDM were resuspended in three 6.5 ml lots of incubation buffer (pH 7.2). After incubating at 30 °C for 10 min to de-energise, glucose was added to two of the incubation systems to a final concentration of 0.2 % (w/v). After a further 15 min Val-Gly_2 was added to each incubation system to a final concentration of 1 mM. Exactly 30 min later CCCP was added to one of these incubation systems containing glucose to a final concentration of 1 $\mu g.ml^{-1}$. Timed samples were taken at 15 min intervals from each incubation system over 60 min from the time the Val-Gly_2 was added. At the end of this hour the cells were removed from each system by centrifuging at 7000 g for 5 min at 4 °C. The supernatants were filtered through a 0.22 µM Millipore filter and more peptide was added to each to a final concentration of 1 mM assuming all the original peptide had been consumed. Timed samples were then taken over a further 60 min to assess extracellular peptidase activity.

These results are consistent with the uptake of Val-Gly_2 being energy dependent. In the absence of glucose, Val-Gly_2 levels in the cell suspension remain constant for the first 30 min of incubation. During the next 30 min of incubation supernatant peptide levels declined rapidly. The CE profiles show that this decline in Val-Gly_2 levels is due to tripeptidase cleavage since from 30 min a peak corresponding to Gly-Gly begins to appear (data not shown). This release of peptidase into the medium must be due to autolysis of cells, presumably a consequence of the absence of an energy source.

When cell suspensions were incubated in the presence of Val-Gly_2, glucose and CCCP for 30 min, Val-Gly_2 uptake was completely inhibited.

4.2.6 Assessment of the effect of potential competitors on Val-Gly transport.

From the observation that growth of certain *L. cremoris* strains on dipeptides as sole sources of essential amino acids could be inhibited by the addition of structurally related dipeptides, whereas no such inhibition was seen with *L. lactis* strains, Law (1977) concluded that there were significant strain differences in the mechanism of lactococcal peptide utilisation.
To confirm the observations of Law with respect to *L. cremoris* strains, it was decided to study the effect of potential competitors upon the uptake of Val-Gly by strain E8. The results obtained when cell suspensions of *L. cremoris* E8 were incubated with Val-Gly in the presence of a fourfold higher concentration of one of two other dipeptides are presented in Table 4.2.6.1 and Figure 4.2.6.1. Val-Gly uptake was severely reduced in the presence of either Leu-Gly or Glu-Ala. This finding is consistent with previous studies indicating a single, broad specificity transport system for dipeptides in *L. cremoris*. and provides further evidence that the utilisation of Val-Gly by *L. cremoris* cell suspensions reflects transport of the dipeptide as such, rather than after hydrolysis to free amino acids.

4.2.7 Investigation of possible nutritional regulation of di-/tripeptide transport in whole cells of *L. cremoris* E8

In all the peptide uptake experiments reported to date, the cells used have been grown in chemically defined media supplemented with free valine. In using such cells to determine peptide uptake rates, the assumption is made that the transport system is constitutively expressed in the absence of the peptide and that this expression is not significantly altered on exposure to the peptide. In order to assess the possibility that expression of the di-/tripeptide transport system might be nutritionally regulated, a comparison was made of the rates of Val-Gly_2 transport in cells grown in a medium containing Val-Gly_2 as the valine source and in cells grown on the usual CDM + valine. The results of the transport experiments carried out with each of these cell types (Table 4.2.7.1) show that Val-Gly_2 uptake by Val-Gly- adapted cells was not significantly different to the rate observed when valine-grown cells were used. These results suggest that the di-/tripeptide transport system is constitutively expressed.

Table 4.2.6.1 The effects of potential competitors on Val-Gly uptake by washed cell suspensions of *L.cremoris* E8.

Peptides	Val-Gly Removal Rate by Cell Suspensions ($nmol.h^{-1}.mg^{-1}$)	Val-Gly Removal Rate due to Peptidase Activity ($nmol.h^{-1}.mg^{-1}$)	Net Rate of Val-Gly Uptake by Cells ($nmol.h^{-1}.mg^{-1}$)
1 mM Val-Gly	872.7	0	873
1 mM Val-Gly 4 mM Leu-Gly	344.8	0	345
1 mM Val-Gly 4 mM Glu-Ala	356.6	0	357

L.cremoris E8 cells grown in CDM were resuspended in 6.5 ml of incubation buffer. After incubating at 30 °C for 10 min to de-energise, glucose was added to a final concentration of 0.2 % (w/v). After a further 15 min Val-Gly, and either Leu-Gly or Glu-Ala, were added to final concentrations of 1 mM and 4 mM respectively. Timed samples were then taken at 15 min intervals over the next 60 min. At the end of this hour the cells were removed by centrifugation at 7000 g for 5 min at 4 °C. The supernatant was subsequently filtered through a 0.22 µM Millipore filter, and a further aliquot of peptide was added. Timed samples were then taken at 20 min intervals over a further hour to assess extracellular peptidase activity.

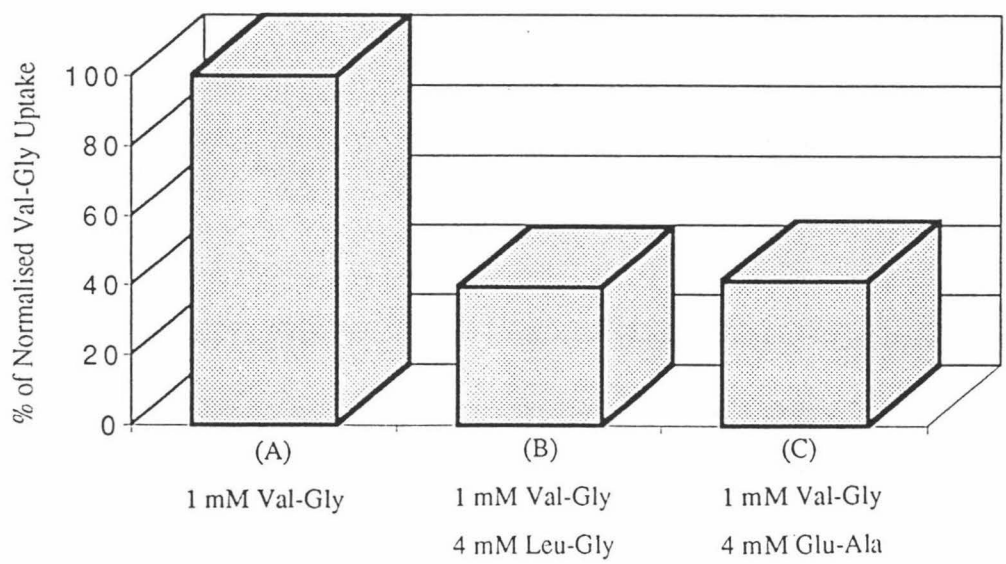


Figure 4.2.6.1 Graphical representation of the data presented in Table 4.2.6.1. Rates of Val-Gly uptake in the presence of Leu-Gly or Glu-Ala have been expressed as a percentage of the control rate.

Table 4.2.7.1 The effect of different growth media upon the rate of transport of Val-Gly_2 by *L. lactis* subsp. *cremoris* E8.

Medium cells grown up in.	Val-Gly_2 Removal Rate by Cell Suspensions (nmol h^{-1} mg^{-1})	Val-Gly_2 Removal Rate due to Peptidase Activity (nmol h^{-1} mg^{-1})	Net Rate of Val-Gly_2 Uptake by Cells (nmol h^{-1} mg^{-1})
CDM + Val	895.0	0	895.0
CDM+Val-Gly_2	992.0	0	992.0

4.2.8 Preliminary studies into the utilisation of β-casein oligopeptides by *L. cremoris* E8.

Studies of whole cell peptide utilisation have used as substrates either a series of peptides homopolymers such as Ala_X series (Smid, 1991; Kunji *et al.*, 1993), a series of synthesised homologous peptides (present study; Rice *et al.*, 1978), or a collection of unrelated peptides which contain amino acids essential to the growth of lactococci and which were available commercially (Law, 1978; Tynkkynen *et al.*, 1993; Yu, 1994).
While such peptides have been useful for characterising the size limits of the lactococcal transport systems, and for determining relative rates of activity of the di-/tripeptide transport system and the oligopeptide transport system, they are chemically quite different from the peptide substrates that the lactococci metabolise in milk. An important question therefore remains about how relevant the knowledge that has been obtained about lactococcal peptide transport from studies using synthetic peptides is to *in vivo* casein utilisation.

To this end, experiments were carried out to assess the ability of *L. cremoris* E8 to utilise the β-casein derived oligopeptides, KAVPYPQ (peptide 1), QEPVLGPVRGPFPIIV (peptide 2), and RDMPIQAFLLY (peptide 3).
These three peptides are produced from the proteolytic cleavage of the C-terminal end of β-casein by the cell wall-associated proteinase (Reid *et al.*, 1991a). Purified quantities of each peptide were obtained for the present research by carrying out repeated HPLC separations of a 3 h proteinase digest of purified β-casein

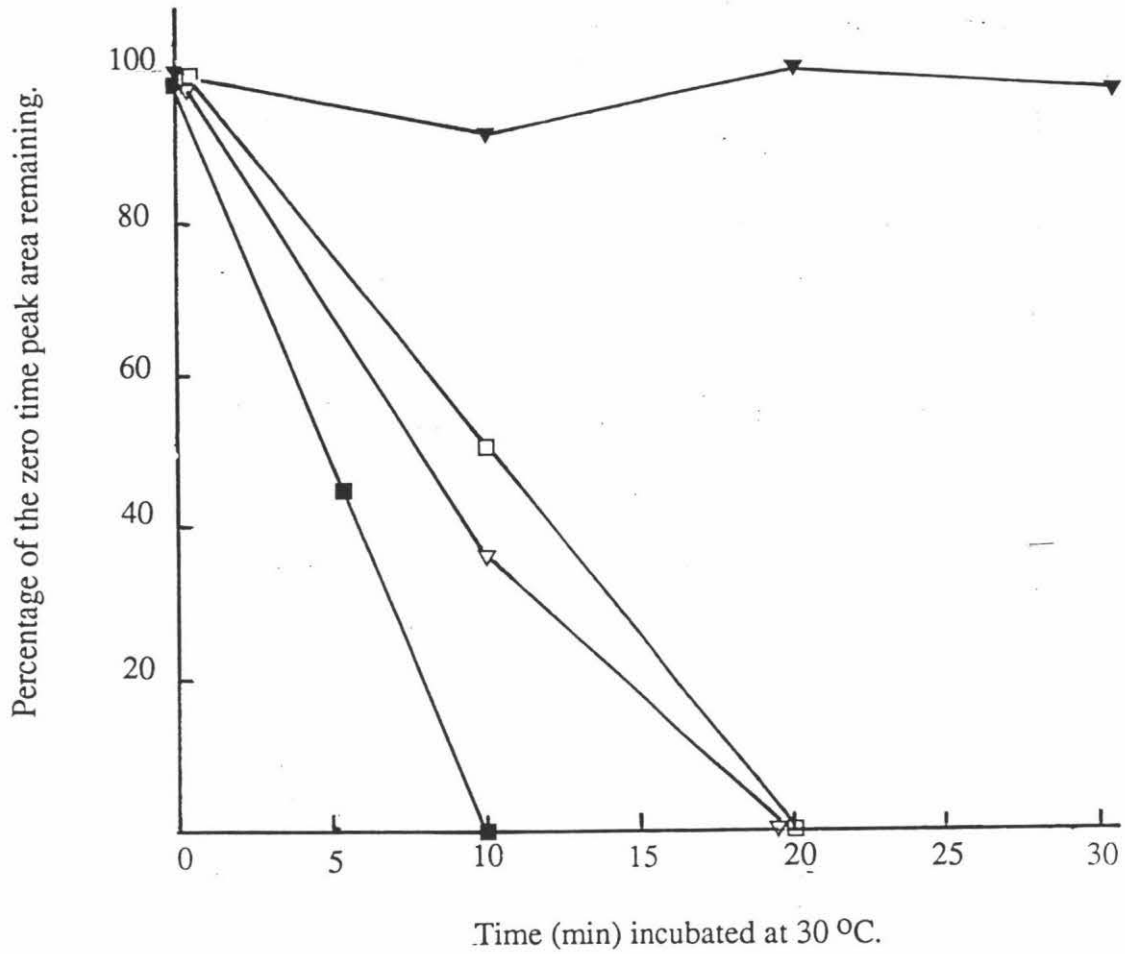


Figure 4.2.8.1 Graph showing the decrease in β-casein oligopeptides when incubated with cell suspensions of *L. cremoris* E8. Refer to text for details.

Peptide 1	(KAVPYPQ) in the presence of glucose	(■)
Peptide 2	(QEPVLGPVRGPFPIIV) in the presence of glucose	(□)
Peptide 3	(RDMPIQAFLLY)in the presence of glucose	(▽)
Peptide 2	in the absence of glucose	(▼)

(Section 2.2.9). The ability of washed cell suspensions to utilise these peptides was then determined by carrying out uptake studies (Section 2.2.10).

A summary of the results obtained for the rates of utilisation of these peptides is shown in Figure 4.2.8.1. Due to the small amounts of the three substrates available for use, it was not possible to standardise the areas of the capillary electrophoresis peaks corresponding to these peptides and express the rate of peptide removal from the supernatant in terms of nmol. min^{-1}. Instead the areas of the peaks in the timed samples are expressed as a percentage of the area of the same peak in the zero time sample.

The ability of *L. cremoris* E8 to utilise the 7 residue peptide 1 is consistent with earlier results presented in this study which showed this strain could rapidly utilise peptides containing up to 8 residues (Section 4.2.3). Utilisation of this peptide appears to involve transport without the need for prior hydrolysis. Examination of the CE profiles obtained when determining peptide 1 uptake by strain E8 (Figure 4.2.8.2) show the appearance of a second peak, which has been determined by use of a standard to correspond to the free amino acid tyrosine (data not shown). The side chain of this amino acid contains an aromatic ring which will be detectable at a wavelength of 200 -220 nm. The absence of further peaks corresponding to the peptides KAVP and PQ in the samples taken from the supernatant of the whole cell suspensions, suggests that the tyrosine has been derived by complete hydrolysis of peptide 1 by intracellular peptidases after the peptide has been transported, rather than by extracellular peptidases.

The 16 residue peptide 2 and the 11 residue peptide 3 are also removed from the supernatant although at a slower rate than peptide 1. The absence of peaks corresponding to possible cleavage products of peptides 2 and 3 in the CE profiles from the uptake studies carried out using these peptide substrates, suggests that they too can be taken up by *L. cremoris* E8 without prior hydrolysis into smaller peptides. To provide further evidence towards the proposal that these large oligopeptides are transported into cells, *L. cremoris* E8 was incubated with peptide 2 in the absence of glucose. No significant decrease in the supernatant concentration of this peptide was observed, indicating that utilisation of peptide 2 was an active process.

Although these preliminary experiments indicate that *L. cremoris* E8 can utilise peptides 1, 2 and 3, few conclusions can be made about their relative rates of uptake. The relative absorbance of the Val-$(Gly)_x$ peptide series at 200 nm was

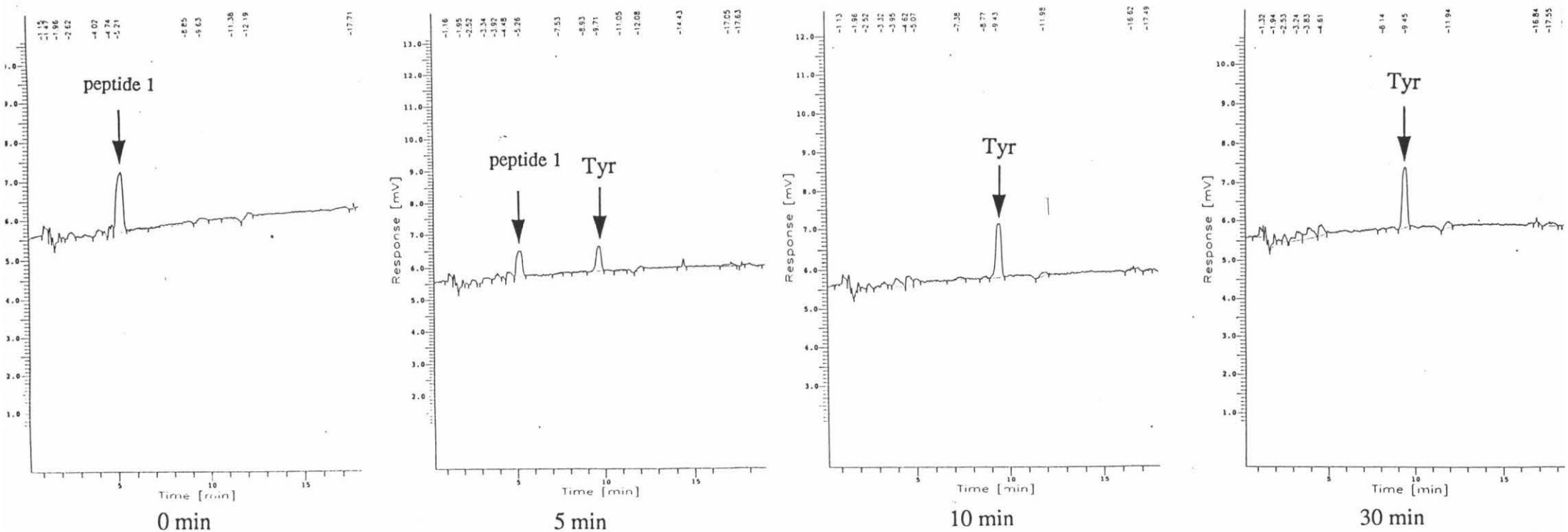


Figure 4.2.8.2 Capillary electrophoresis profiles from a time course study of peptide 1 (KAVPYPQ) uptake by glycolysing cell suspensions of *L. lactis* subsp. *cremoris* E8. A 2.5 sec injection was made onto a 60 cm Freezone column. Peptides were eluted by running in 20 mM sodium citrate pH 2.5 at 30 μV for 20 min.

directly proportional to the number of peptide bonds present. It was therefore possible to make a qualitative assessment of the relative rates of uptake of the various peptides solely by comparing the areas of the CE peaks corresponding to these peptides. The presence of tyrosine in peptides 1 and 3 prevents such a comparison with the β-casein peptides as this residue will contribute to the absorbance, and therefore peak areas, of these peptides.

It is likely however that the rates of transport of peptides 2 and 3 are quite slow. While the CE profiles of the timed samples show that peptides 2 and 3 have disappeared from the supernatant within 20 min of being incubated with whole cells, the concentration of peptide used is much lower than the 1 mM levels used of the Val-$(Gly)_X$ peptides, while the density of cells about 10 fold higher. An attempt was made to reassess uptake of these peptides using a cell suspension density much more comparable to that used in the uptake studies with the valine-peptides. However, this attempt was unsuccessful and exhausted the stocks of the β-casein peptides. Because of the time that would be required to purify more of these peptides, it was decided not to pursue these preliminary studies further.

4.3 *Lactococcus lactis* 920

From studies on peptide utilisation by a range of *L. lactis* and *L. cremoris* strains, Law (1977) drew a general distinction between these two groups, concluding that *lactis* strains hydrolysed dipeptides extracellularly while *cremoris* strains did not. This distinction has been shown to apply to the *lactis* strain 1403 and the *cremoris* strain E8 in the present study. However in view of the high level of intracellular leakage found with 1403, it was important to include another *lactis* strain as part of the present comparative study. *L. lactis* 920 was selected from the *lactis* strains available for research on the basis of the results obtained from preliminary studies into the susceptibility of this strain to leakage of intracellular enzymes (Section 4.3.1) and its ability to grow in chemically defined medium (Section 4.3.2).

4.3.1 Assessment of leakage of intracellular enzymes from *L. lactis* 920 cells

The discovery that *L. lactis* 1403 cell suspensions were prone to autolysis and release of intracellular peptidases, made this strain unsuitable for use in peptide uptake studies. It was therefore important to assess the level of intracellular peptidase leakage with the 920 strain. The results obtained from determining aldolase levels in the supernatant of incubated *L. lactis* 920 washed cell suspensions, previously grown in CDM, are presented in Table 4.3.1.1.

Table 4.3.1.1. Assessment of intracellular adolase leakage from incubating whole cells of *L. lactis* subsp. *lactis* 920. Cells were incubated under the same conditions as used in the peptide uptake experiments, except for the omission of peptide.

Time (min)	Aldolase Activity (μmol min^{-1} ml^{-1})		
	(A) Supernatant	(B) French Press Extract	A/B x 100 (%)
0	8.76×10^{-4}	0.169	0.52
30	1.58×10^{-3}	0.214	0.59
60	1.23×10^{-3}	0.267	0.81

(Aldolase levels were determined using the method of Crow *et al.*, 1982)

These results show supernatant aldolase levels to be less than one percent of the total cellular adolase activity. This level is insignificant when compared to the 38 percent found for *L. lactis* 1403 (Table 4.1.4.1), and indicates that this strain is not predisposed to leakage of its internal peptidase complement under the experimental conditions used for the uptake studies.

4.3.2 Growth and peptidase activities of *L. lactis* 920 in chemically defined medium.

As part of a comparative study of lactococcal strain differences, *L. lactis* 920 was grown in CDM, and the peptidase levels of these cells determined. The results are shown in Figure 4.3.2.1 and Table 4.3.2.1. *L. lactis* 920 grows well in CDM, with a maximum growth rate of 0.693, and reached a final OD_{540nm} of 6.0. The final OD obtained is much higher than the corresponding values found for *L. lactis* 1403 and *L. cremoris* E8 cultures grown under the same conditions.

The intracellular peptidase levels determined for strain 920 cells grown on CDM are similar to those of strains 1403 and E8.

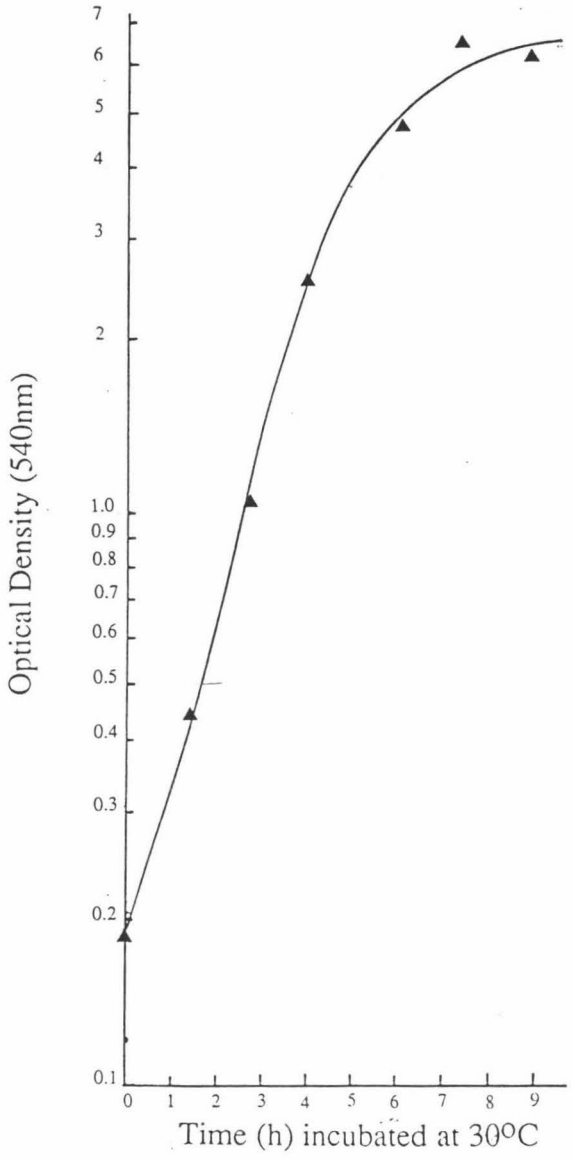


Figure 4.3.2.1 Growth of *L. lactis* subsp *lactis* 920 in chemically defined medium, in which all amino acids are supplied in their free state, and containing 1.5% (w/v) lactose. Cells were grown through two passages of this medium before measurements were taken.

Table 4.3.2.1 Peptidase activities of a French Press extract of *L.lactis* 920 cells grown in chemically defined media. Peptidase activities are expressed as $\mu mol.min^{-1}.mg^{-1}$ protein.

Media	Dipeptidase Ala-Ala[a]	Tripeptidase Leu-Gly2	Prolidase Leu-Pro	Pro/imino-peptidase Pro-Ala	Lys-amino peptidase Lys-*p*NA	X-prolyl dipeptidyl aminopeptidase Gly-Pro-AMC
920	2.98	0.642	0.383	0.012	0.089	0.160

[a] substrate used for the assay of peptidase activities.

4.3.3. Uptake of Val-$(Gly)_X$ peptides by *L. lactis* 920

The net rates of uptake of the synthetic peptides Val-Gly, Val-Gly_2. Val-Gly_3, Val-Gly_4 and Val-Gly_7 by washed cell suspensions of *L. lactis* 920 are presented in Table 4.3.3.1.

As anticipated from the determination of the extent of adolase leakage, no detectable hydrolysis of Val-Gly_2 and Val-Gly_3 by supernatant peptidase activity was found. However a low rate of Val-Gly hydrolysis was found which presumably reflects some dipeptidase leakage. Due to the high level of intracellular dipeptidase activity (Table 4.3.2.1), a leakage rate of even one percent may result in detectable hydrolysis.

Whereas the relative rates of uptake of the Val-$(Gly)_X$ peptides determined for *L. cremoris* E8 (Table 4.2.3.1) were very similar, the net rates of uptake for the same peptides by *L. lactis* 920 show significant differences.
Val-Gly_2 uptake rates by washed cell suspensions of *L. lactis* 920 were almost three times as high as those for Val-Gly uptake, suggesting that the di-/tripeptide transport system of strain 920 has a greater activity for tripeptides than dipeptides.
Previous studies on the substrate specificity of the di-/tripeptide transport system of *L. cremoris* ML3 (Smid, 1991) have, in contrast, reported dipeptide transport to be significantly more rapid than tripeptide transport.
Secondly, rates of oligopeptide uptake were comparable to those of di- and tripeptide uptake. While this result is in agreement with the peptide uptake rates determined for *L. cremoris* E8, it is in variance with previous research into the relative activities of the di-/tripeptide and oligopeptide transport systems which suggested that oligopeptide transport was significantly slower than di-/tripeptide transport (Smid, 1991).
However, whereas the rates of uptake of Val-Gly_3, Val-Gly_4 and Val-Gly_7 by *L. cremoris* E8 cell suspensions were very similar, oligopeptide uptake rates by *L. lactis* 920 cell suspensions decreased as peptide size increased. Due to difficulties with Val-Gly_9 synthesis (Section 3.1.4), it was not possible to demonstrate whether this observed inverse relationship between peptide size and rate of uptake continued to hold when a decapeptide was used as a substrate.

Another strain difference observed in this study concerns Val-Gly_7 uptake by washed cell suspensions. Capillary electrophoresis profiles obtained when determining Val-Gly_7 uptake by *L. lactis* 920 are presented in Figure 4.3.3.1a.

Table 4.3.3.1 Net Rates of uptake of Val-$(Gly)_x$ peptides by *L. lactis* subsp *lactis* 920

Peptide	Peptide Removal Rate by Cell Suspensions (nmol h^{-1} mg^{-1})	Peptide Removal Rate due to Peptidase Activity (nmol h^{-1} mg^{-1})	Net Rate of Uptake by Cells (nmol h^{-1} mg^{-1})
Val-Gly	695.8	115.5	580
$Val\text{-}Gly_2$	1453	0	1453
$Val\text{-}Gly_3$	914.8	0	915
$Val\text{-}Gly_4$	677.0	ND[a]	677
$Val\text{-}Gly_7$	451.0	ND	451

Details of the experimental procedure are given in the legend to table 4.2.3.1. To preserve substrates, peptidase controls were not carried out with the larger Val-(Gly)x peptides. Any extracellular hydrolysis of these substrates should be obvious from their CE profiles as discussed in Section 4.1.3.

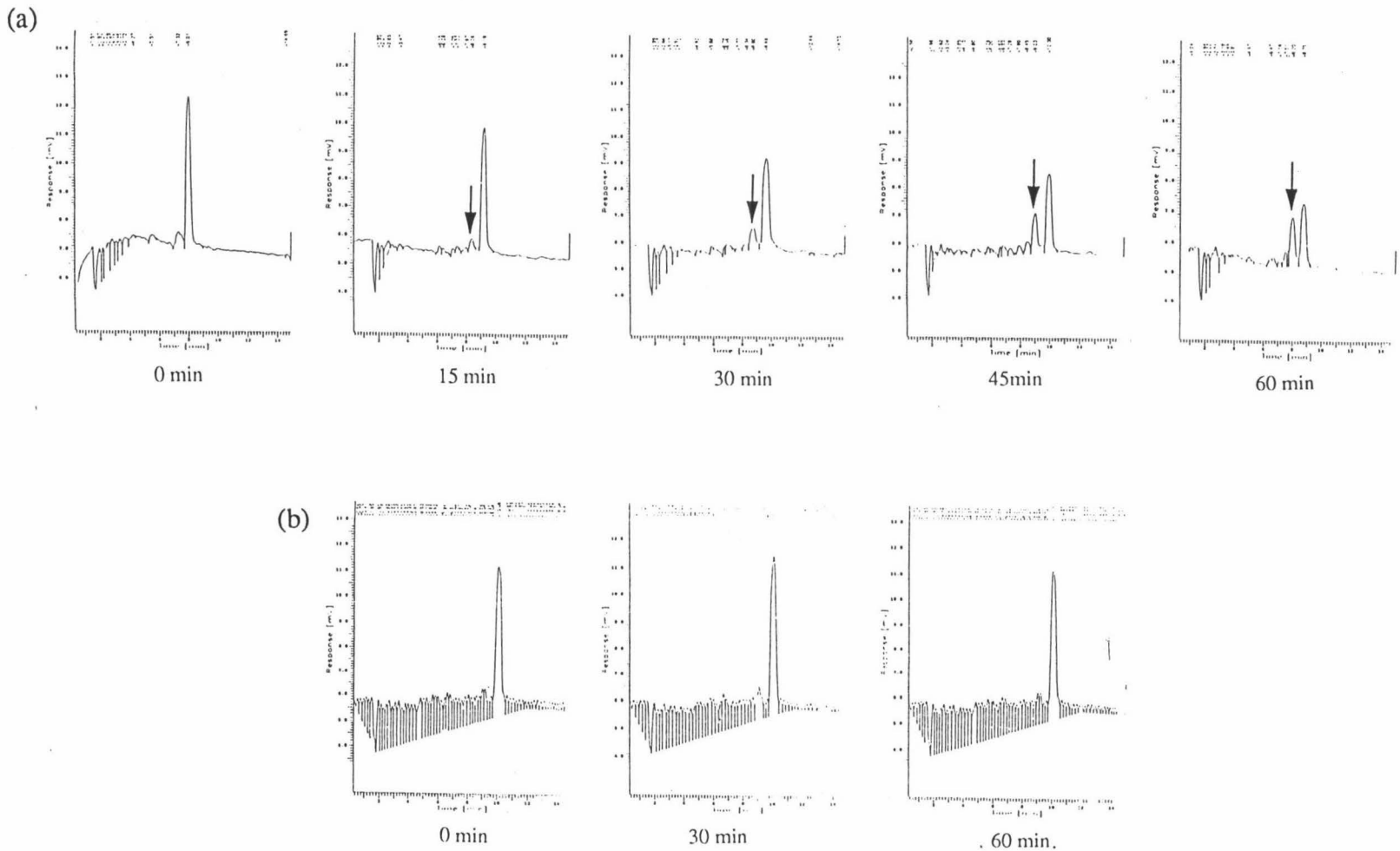


Figure 4.3.3.1 Capillary electrophoresis profiles from a time course study of Val-Gly7 uptake by glycolysing cell suspensions of *L. lactis* subsp. *lactis* 920. (a) Val-Gly7 removal from the supernatant in the presence of whole cells of *L. l actis* 920. (b) Val-Gly7 removal from the cell-free supernatant by extracellular peptidases. The arrow marks the appearance of a peptide whose identity was not determined. A 2.5 sec injection was made onto a 72 cm Freezone column. Peptides were eluted by running in 20 mM sodium citrate pH 2.5 at 30 kV for 30 min.

Examination of these profiles shows the appearance of a second peak (identified by an arrow) in addition to that corresponding to Val-Gly$_7$. This peak was absent from the CE profiles obtained when measuring the uptake of Val-Gly$_7$ by *L. cremoris* E8 (Figure 4.2.3.2). It was possible that the second peak present in the *L. lactis* 920 CE profiles corresponded to a product of Val-Gly$_7$ cleavage by an extracellular peptidase (as found in the case of *L. lactis* 1403 when measuring Val-Gly$_2$ uptake - Section 4.1.3). To test the possibility that Val-Gly$_7$ removal from the supernatant of *L. lactis* 920 suspensions could be due to the activity of a leaked aminopeptidase or general endopeptidase, the experiment was repeated with a peptidase control. The results of this control demonstrated that once the cells were removed from the incubation buffer, there was no further decrease in t he area of the Val-Gly$_7$ peak (Figure 4.3.3.1b).
This result demonstrates that the hydrolysis of Val-Gly$_7$ is dependent upon the presence of cells, which would not be the case if peptide hydrolysis was due to extracellular peptidase activities. It is more likely that Val-Gly$_7$ is being transported intact, hydrolysed internally and one of the products of this cleavage transported back into the supernatant.
Due to the minute amounts of material available, it was not possible to identify this second peak.

4.3.4 Utilisation of Val-(Gly)$_x$ peptides as a source of valine for growth of *L. lactis* 920 in chemically defined media.

The growth of *L. lactis* 920 was investigated in chemically defined media, which differed in their source of the essential amino acid valine. One medium contained valine as the free amino acid, while three other media supplied valine as an equivalent concentration of either Val-Gly, Val-Gly$_2$ or Val-Gly$_3$.

Experiments in which *L. lactis* 920 was grown in CDM over a range of valine concentrations, showed that at 0.22 mM there was a marked reduction in the growth rate of the culture (Table 4.3.4.1), which is considerably lower than the growth rate limiting concentration (0.85 mM) found for *L. cremoris* E8. This growth rate limiting concentration of free valine was assumed to be the same for when valine was supplied as a peptide form. (Note that this experiment was carried out before analysis of the growth rates of the *cremoris* strain E8 on different nitrogen sources indicated that this assumption was not valid).

Chemically defined media were prepared containing either valine or Val-(Gly)$_x$ peptides at 0.22 mM. Amino acid analysis of these media

Table 4.3.4.1 Determination of the growth rates of *L. lactis* subsp. *lactis* 920 on chemically defined media supplemented with different concentrations of the essential amino acid valine.

Concentration of valine in the CDM ($mg.l^{-1}$)	Concentration of valine in the CDM (mM)	Growth Rate.
400[a]	.3.42	0.630
200	1.71	0.690
100	0.85	0.670
50	0.42	0.401
25	0.22	0.224
0	0	0

[a] This is the concentration of valine used in the growth experiments carried out previously with *L. lactis* 1403.

L. lactis 920 cells were grown through two generations of the media of interest before growth measurements were taken.

(Table 4.3.4.2) showed the available valine concentration in each to be comparable. The growth curves obtained when strain 920 cells were grown on each of these media are presented in Figure 4.3.4.1. Growth was strong in all media, demonstrating that strain 920 can utilise dipeptides, tripeptides and oligopeptides as sources of the essential amino acid valine.

As with strain E8, no conclusions can be made about the relative activities of the di-/tripeptide transport system and the oligopeptide transport system. The maximum growth rates of strain 920 in CDM in which valine was supplied as a peptide are evidently much higher than when valine was supplied as an amino acid. This result indicated that 0.22 mM is not the growth rate limiting concentration for valine when it was supplied as a Val-$(Gly)_X$ peptide. Due to the time it would take to determine the real growth rate limiting concentration of the various valine peptides, this study was not repeated

However, the ability of *L. lactis* 920 cells to grow rapidly on the peptide - containing media, does provide indirect evidence that these peptides are being taken up without prior hydrolysis. If extracellular hydrolysis did precede transport, the released valine would be subject to competition for transport by

Table 4.3.4.2 Analytically determined valine concentrations in the various chemically defined media used in growth experiments with *L. lactis* 920.

Media	nmol Val $100\mu l^{-1}$ of Media
CDM - Val	0.0
CDM + 0.22 mM Val	22.0
CDM + 0.22 mM Val-Gly	25.7
CDM + 0.22 mM Val-Gly_2	26.0
CDM + 0.22 mM Val-Gly_3	29.0

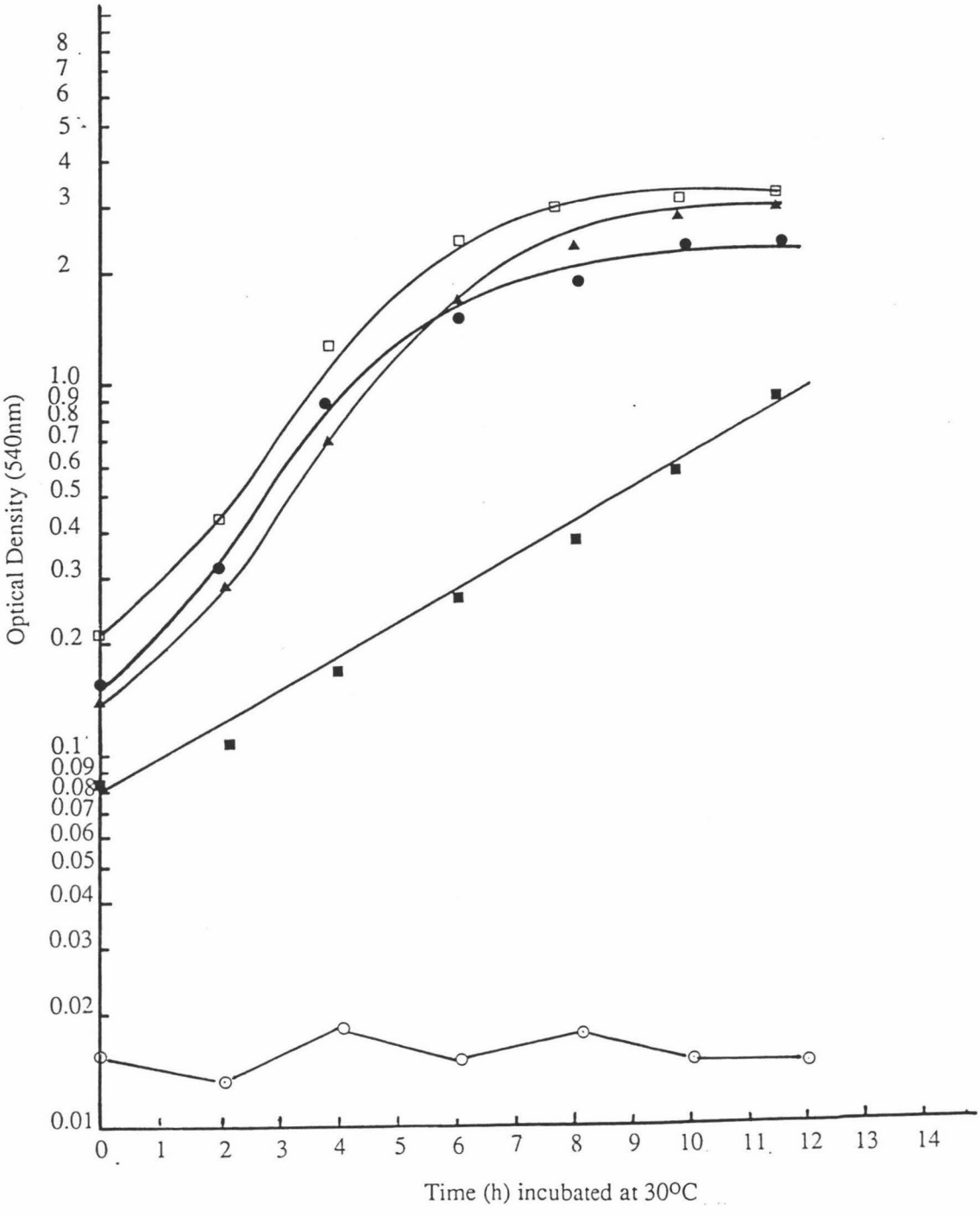


Figure 4.3.4.1. Growth of *L.lactis* subsp *lactis* 920 in chemically defined media supplemented with 1.5 % (w/v) lactose, and different valine sources.

Chemically defined medium (CDM) without a valine source	(○)
CDM containing 0.22 mM valine	(■)
CDM in which free valine is replaced by 0.22 mM Val-Gly	(●)
CDM in which free valine is replaced by 0.22 mM $Val\text{-}Gly_2$	(▲)
CDM in which free valine is replaced by 0.22 mM $Val\text{-}Gly_3$	(□)

Cells were grown through two passages of the medium of interest before measurements were taken.

leucine and isoleucine in the medium. Cell growth rates would then be much more comparable to those seen for cultures grown in CDM supplemented with an equivalent concentration of free valine.

4.3.5 Energy dependence of oligopeptide uptake in *L. lactis* 920

To provide further evidence to support the theory that the observed oligopeptide utilisation by washed cell suspensions of *L. lactis* 920 was due to the active transport of these peptides into cells by the oligopeptide transport system, experiments were carried out to show this process to be energy-dependent. The results obtained when *L. lactis* 920 was incubated with Val-Gly_3 in the presence or absence of glucose are shown in Table 4.3.5.1.
In the absence of glucose as an energy source, supernatant Val-Gly_3 levels remained constant over the time period measured, which is consistent with the conclusion that oligopeptide uptake is an active process.

The effect of the ATPase inhibitor vanadate was also investigated as the oligopeptide transport system has been shown to be coupled to ATP hydrolysis (Kunji *et al.*, 1993).

Table 4.3.5.1 Demonstration of the energy dependence of Val-Gly_3 uptake by *L. lactis* subsp. *lactis* 920

Parameter	Val-Gly_3 Removal Rate by Cell Suspensions (nmol h^{-1} mg^{-1})	Val-Gly_3 Removal Rate due to Peptidase Activity (nmol h^{-1} mg^{-1})	Net Rate of Val-Gly_3 Uptake by Cells (nmol h^{-1} mg^{-1})
+ glucose	1085.8	0	1086
- glucose	0	0	0

The use of vanadate as a possible inhibitor of the oligopeptide transport system poses a problem since vanadate also inhibits glycolysis which provides the usual ATP source for lactococci. The *lactis* strains of lactococci can, however, use arginine degradation by the arginine deaminase(ADI) pathway as an alternative

mechanism for ATP production (Crow and Thomas, 1982). In this pathway, arginine is converted into ornithine, ammonia, and CO_2 to yield one mol of ATP for every mol of arginine catabolised. Previous research (Kunji *et al.*, 1993) has shown that the activity of the ADI pathway is barely affected by vanadate at concentrations as high as 0.5 mM. Val-Gly_7 uptake in the presence of vanadate was assessed using whole cell suspensions of *L. lactis* 920 (Table 4.3.5.2). Two different concentrations of vanadate were used, one (0.5 mM) identical to that found to be inhibit oligopeptide uptake by Kunji *et al.* (1993), and the other (5 mM) ten times higher in case of strain differences in the sensitivity to vanadate. Because *L. cremoris* strains lack essential enzymes in the ADI pathway, it was not possible to carry out these experiments with strain E_8.

The rates of uptake of Val-Gly_7 in the cell suspensions without vanadate are lower than those found previously for this strain when glucose was the energy source (451 $nmol.h^{-1}mg^{-1}dwc$).This is probably due to the considerably lower rates of ATP production by the ADI pathway relative to those attained using glucose as an energy source. Even at 5 mM, vanadate had no effect upon Val-Gly_7 uptake by cell suspensions of *L. lactis* 920. These results are not in agreement with previous work which showed that 0.5 mM vanadate completely inhibited tetra-alanine uptake by cell suspensions of *L. cremoris* ML_3 (Kunji *et al.*, 1993). It is difficult to explain this discrepancy as the experimental conditions used in this current study are almost identical to that carried out by Kunji *et al.* (1993).

It was decided not to pursue this work with vanadate, since an alternative approach to establishing the involvement of the oligopeptide transport system in oligopeptide utilisation subsequently became available (Chapter 5)

4.3.6 The effects of potential competitors upon Val-Gly uptake by *L. lactis* subsp. *lactis* 920.

Law (1977) concluded from the observation that the growth of *L. lactis* 2017 upon dipeptides as sole sources of amino acids, was not inhibited by structurally related peptides, that *L. lactis* strains hydrolyse dipeptides extracellularly into amino acids for transport.

To confirm this observation Val-Gly uptake rates by whole cell suspensions of strain 920 were determined in the presence of a fourfold excess of Leu-Gly or Glu-Ala (Table 4.3.6.1 and Figure 4.3.6.1). It was found that Val-Gly uptake was

Table 4.3.5.2 The effect of the ATPase inhibitor vanadate on Val-Gly_3 uptake by whole cell suspensions of *L. lactis* subsp *lactis* 920.

Parameter	Val-Gly_7 Removal Rate by Cell Suspensions ($nmol.h^{-1}.mg^{-1}$)	Val-Gly_7 Removal Rate due to Peptidase Activity ($nmol.h^{-1}.mg^{-1}$)	Net Rate of Val-Gly_7 Uptake by Cells ($nmol.h^{-1}.mg^{-1}$)
25 mM Arg No Vanadate	204	0	204
25 mM Arg 1 mM Vanadate	210.6	0	211
25 mM Arg 5 mM Vanadate	194	0	194

This experiment was based on the work of Kunji *et al.*, 1993. Milk grown cells of strain *L. lactis* 920 were grown through one passage of UBM, followed by two generations of CDM-ARG (this is chemically defined medium which has been supplemented with 0.5% lactose and 25 mM arginine). When the cells had reached a final A_{540nm} of 1.5, they were harvested by centrifuging at 7000g for 5 min at 4 ^{o}C. After washing with 100 mM MES, pH 6.5, the cells were resuspended up in 6.5 ml of the same buffer for every 10 ml of original culture.
Three separate incubation systems were then set up as follows (concentrations given are the final values in the incubation mixture);

(i) 6.5 ml MES + 25 mM Arg + 1 mM Val-Gly_7

(ii) 6.5 ml MES + 25 mM Arg + 1 mM Val-Gly_7 + 0.5 mM orthovanadate (VO_4).

(iii) 6.5 ml MES + 25 mM Arg + 1 mM Val-Gly_7 + 5 mM VO_4

After incubating the buffered cell suspensions in the presence of vanadate for 10 min at 30 ^{o}C, the arginine was added, followed 15 min later by the peptide. Timed samples were taken at regular intervals over a 120 min period.

Table 4.3.6.1 The effects of potential competitors on Val-Gly uptake by washed cell suspensions of *L.lactis* subsp. *lactis* 920

Peptides	Val-Gly Removal Rate by Cell Suspensions ($nmol.h^{-1}.mg^{-1}$)	Val-Gly Removal Rate due to Peptidase Activity ($nmol.h^{-1}.mg^{-1}$)	Net Rate of Val-Gly Uptake by Cells ($nmol.h^{-1}.mg^{-1}$)
1 mM Val-Gly	617.6	140.5	477
1 mM Val-Gly 4 mM Leu-Gly	476.9	0	477
1 mM Val-Gly 4 mM Glu-Ala	485.3	77.9	407

Details of the experimental procedure are described in the legend to Table 4.2.6.1

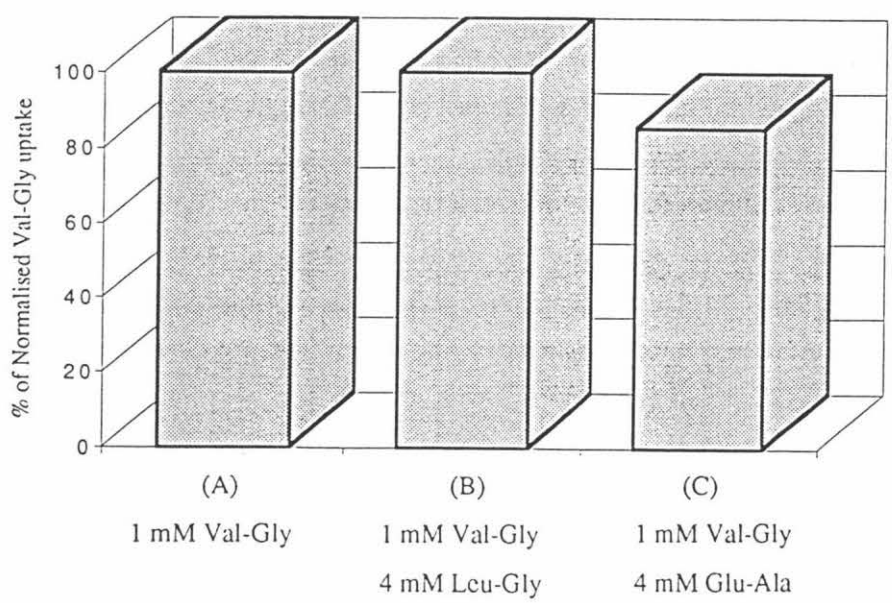


Figure 4.3.6.1 Graphical representation of the data presented in Table 4.3.6.1. Rates of Val-Gly uptake in the presence of Leu-Gly or Glu-Ala have been expressed as a percentage of the control rate.

not inhibited by the presence of Leu-Gly, and only marginally inhibited by the presence of Glu-Ala. These results contrast with the marked inhibition found with strain E8 (Figure 4.2.6.1), but are consistent with Law's (1977) observations that the dipeptide was being cleaved into its constitutive amino acids for transport, rather than being transported intact.

However, results from previous experiments with strain 920 do not support this conclusion. The adolase leakage results (Table 4.3.1.1) provide strong evidence that *L. lactis* 920 is not prone to autolysis and release of intracellular peptidases. This is supported by analysis of the CE profiles obtained when determining Val-Gly_2 uptake by *L. lactis* 920, which do not contain the Gly-Gly peak that acts as an indication of released tripeptidase activity. A very low level of dipeptidase activity was found in the peptidase control carried out when determining the rate of Val-Gly uptake (see Table 4.3.3.1). However, this probably represents an exaggerated estimate of dipeptidase activity in the medium during the measurement of uptake since the peptidase activity is measured at the end of the uptake period when decreasing medium pH might be expected to enhance peptidase leakage. In any case the removal rate of Val-Gly by peptidase activity is only a small fraction of the total uptake rate so it cannot account for the lack of inhibition by structurally related dipeptides with the 920 strain, given that significant inhibition found under the same condition with the E8 strain. Furthermore, if Val-Gly was being hydrolysed into valine and glycine which were then transported as free amino acids, then the growth rate seen with *L. lactis* 920 on CDM + Val-Gly should have been much closer to that seen when cells were grown on CDM + valine (Section 4.3.4).

The other possible explanation for the results is that competition was not seen because *L. lactis* 920 has multiple di-/tripeptide carrier systems of different specificities. This is not in agreement with current research which suggests that lactococci have a single di-/tripeptide system of a broad specificity. Although such a situation is possible it is not probable from the information available from this and other studies. It would be reasonable to assume that even if multiple dipeptide transport systems existed, the transport system for Val-Gly would also have a strong affinity for Leu-Gly as these peptides differ only by one methyl group. Val-Gly uptake should therefore still be inhibited by the presence of excess Leu-Gly. The results from this study however do not demonstrate this.

It was not possible with the time available to investigate these observations further.

4.4 *Lactococcus cremoris* ML3

The ML3 strain of *Lactococcus lactis* is a widely studied strain which has until recently been classified as a member of the *lactis* subspecies on the basis of its ability to ferment arginine. A recent study of homology of chromosomal DNA from several lactococcal strains (Godon *et al.*, 1992) indicated that the ML3 strain has much closer affinity with *cremoris* strains than with *lactis* strains, and so is referred to as *L. cremoris* ML3 in the present study. Most of our current knowledge regarding the mechanisms of peptide transport is drawn from generalisations based on studies of transport mutants of this strain (Smid, 1991; Kunji *et al.*, 1993; Tynkkynen *et al.*, 1993) It was therefore important to include *L. cremoris* ML3 in this current investigation into possible strain differences in peptide utilisation.

4.4.1 Growth and peptidase activities of *L. cremoris* ML3 on chemically defined media

As part of a comparative study of lactococcal strains, *L. cremoris* ML3 was grown in CDM, and the peptidase activities of these cells determined. The results from these experiments are presented in Figure 4.4.1.1 and Table 4.4.1.1. Strain ML3 grew well in CDM with a maximum growth rate of 0.924 and a final OD_{540nm} of 8.5. Both values are higher than those obtained by other strains investigated in this study.

The peptidase activities are mostly similar to those determined for strains 1403, E8 and 920, although the activity of lysyl aminopeptidase is considerably higher than found for the other strains.

4.4.2 Assessment of leakage of intracellular enzymes from *L. cremoris* ML3 cells

Before proceeding with the uptake studies, the susceptibility of this strain to autolysis under the experimental conditions used was assessed. The adolase levels in the supernatant of *L. cremoris* ML3 cell suspensions after incubation in the standard suspension medium are presented in Table 4.4.2.1.

These results demonstrate that enzyme leakage from strain ML3 is negligible.

4.4.3 Uptake of Val-$(Gly)_x$ peptides by *L. cremoris* ML3

The net rates of uptake of the synthetic peptides, Val-Gly, Val-Gly_2 and Val-Gly_3 are presented in Table 4.4.3.1.

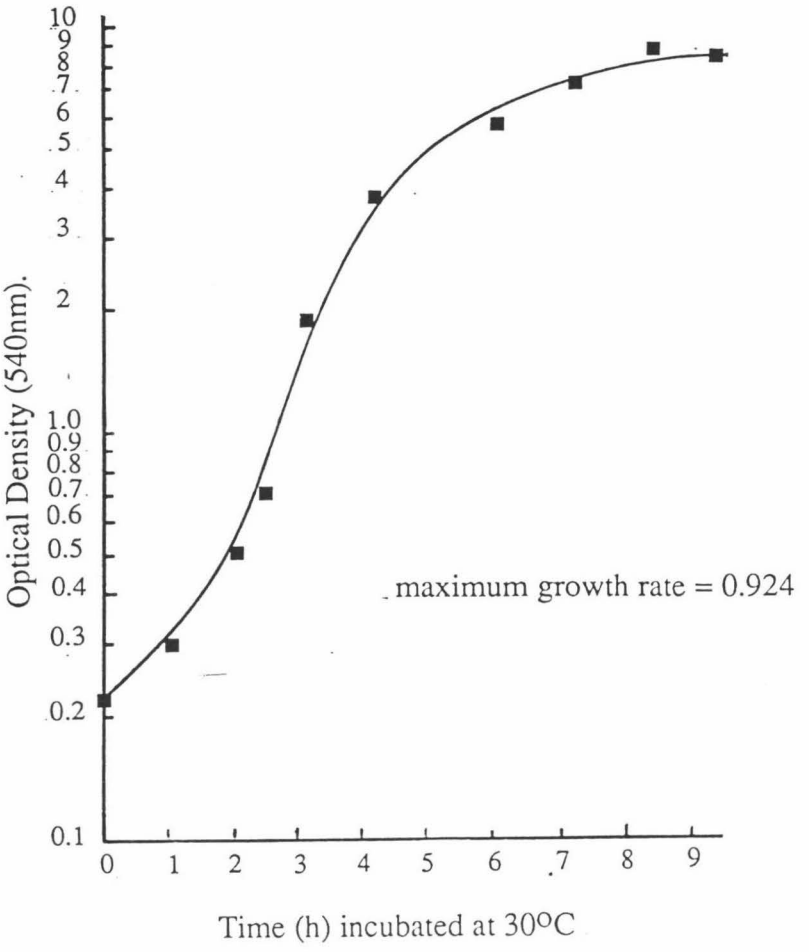


Figure 4.4.1.1 Growth of *L. lactis* subsp. *cremoris* ML3 in chemically defined medium, in which all amino acids are supplied in their free state, and containing 1.5% (w/v) lactose. Cells were grown through two passages of this medium before measurements were taken.

Table 4.4.1.1. Peptidase activities of a French Press extract of *L. cremoris* ML3 grown in chemically defined medium. Peptidase activities are expressed as μmol. min^{-1} mg^{-1} protein.

Media	Dipeptidase Ala-Ala[a]	Tripeptidase Leu-Gly2	Prolidase Leu-Pro	Pro/imino-peptidase Pro-Ala	Lys-amino peptidase Lys-*p*NA	X-prolyl dipeptidyl aminopeptidase Gly-Pro-AMC
ML3	2.12	0.467	0.205	0.016	0.282	0.197

[a] substrate used for the assay of peptidase activities.

Table 4.4.2.1. Assessment of intracellular adolase leakage from incubating whole cells of *L. lactis* subsp. *cremoris* ML3. Cells were incubated under the same conditions as used in the peptide uptake experiments, except for the omission of peptide.

Time (min)	Aldolase Activity (μmol min^{-1} ml^{-1})		
	(A) Supernatant	(B) French Press Extract	A/B x 100 (%)
0	4.46×10^{-3}	0.551	0.81
30	0	0.552	0
60	0	0.490	0.

(Aldolase levels were determined using the method of Crow *et al.*, 1982)

An immediate observation from these results is the very rapid rate of Val-Gly uptake, which is 4.8 times higher than the rate obtained for *L. cremoris* E8 and 6.4 times higher than the rate for *L. lactis* 920.

In contrast to the *lactis* strain 920, the di-/tripeptide transport system of *L. cremoris* ML3 appears to transport dipeptides much more rapidly than tripeptides. This finding is in agreement with the results of earlier studies using this strain (Smid, 1991).

A surprising feature of these results was the failure to observe any uptake of the tetrapeptide, Val-Gly_3. Monitoring of the cell suspension buffer showed a decrease in the pH over the incubation period, which demonstrated that the cell suspensions were metabolising glucose into lactic acid (data not shown).
This result is in conflict with the work of Smid (1991) who found that the tetrapeptide Ala_4 was taken up by cells of this strain, albeit considerably more slowly than Ala_3. An uptake study was therefore carried out with tetraalanine.The results from this uptake experiment (Table 4.4.3.2) indicate that Ala_4 was taken up by the ML3 cells used in this study as found by Smid (1991).
This implies that the failure of ML3 to transport Val-Gly_3 must be because it is not a substrate for the oligopeptide transport system of this strain. As Val-Gly_3 is

Table 4.4.3.1. Net rates of uptake of Val-$(Gly)_x$ peptides by *L. lactis* subsp. *cremoris* ML3

Peptide	Peptide Removal Rate by cell suspensions (nmol h^{-1} mg^{-1})	Peptide Removal Rate due to Peptidase Activity (nmol h^{-1} mg^{-1})	Net Rate of Peptide Uptake by Cells (nmol h^{-1} mg^{-1})
Val-Gly	3701.5	0	3702
$Val\text{-}Gly_2$	1046.5	0	1047
$Val\text{-}Gly_3$	0	0	0

Details for these experiments are given in the legend to Table 4.1.3.2

Table 4.4.3.2 Net rate of uptake of tetra-alanine by *L. lactis* subsp. *cremoris* ML3.

Peptide	Peptide Removal Rate by Cell Suspensions (nmol h^{-1} mg^{-1})	Peptide Removal Rate due to Peptidase Activity (nmol h^{-1} mg^{-1})	Net Rate of Peptide Uptake by Cells (nmol h^{-1} mg^{-1})
Ala_4	461.0	0	461

transported by strains E8 and 920 this result suggests that differences must exist in the substrate specificities of the oligopeptide transport systems of different strains. This has important implications to any attempt to make generalisations about peptide transport from the results of the study of one strain alone (see Chapter 6).

Comparison of the rate of tetra-alanine uptake with the rates of Val-Gly and Val-Gly_2 uptake supports previous research showing the oligopeptide system in ML3 to be much less active than the di-/tripeptide system (Smid, 1991; Kunji et al., 1993).

Due to time constraints it was not possible to confirm the results of these uptake studies by carrying out growth rate studies with ML3 in defined media in which valine was supplied as either Val-Gly, Val-Gly_2 or Val-Gly_3.

4.4.4 The effects of potential competitors upon Val-Gly uptake by *L. lactis* subsp. *cremoris* ML3.

Val-Gly uptake by whole cell suspensions was assessed in the presence of a fourfold excess of two other dipeptides (Table 4.4.4.1 and Figure 4.4.4.1). The results show Val-Gly uptake to be significantly reduced in the presence of either Leu-Gly or Glu-Ala, which is in agreement with Law's observations on competition between dipeptides for uptake by *L. cremoris* strains, and supports the conclusion that transport of dipeptides occurs without prior hydrolysis.

4.5 *Lactococcus cremoris* AM2

Within the *cremoris* group of starter bacteria, Law (1977) found that there was much variation between strains in their ability to utilise dipeptides as sources of essential amino acids. While *L. cremoris* E8 and *L. cremoris* HP grew as well on dipeptides as they did on the equivalent amino acids, other strains such as *L. cremoris* AM2 and *L. cremoris* US3 grew poorly when essential amino acids were substituted for by dipeptides. These differences were inferred to be due to variations in the rates of uptake of the dipeptides by the strains concerned, as all had similar intracellular dipeptidase activities.

Further study of peptide utilisation by *L. cremoris* AM2 (Law, 1978), showed that the poor growth of this strain on dipeptides as sole sources of essential amino acids correlated with a slow rate of uptake of radiolabelled dipeptides. The same study also

Table 4.4.4.1 The effects of potential competitors on Val-Gly uptake by washed cell suspensions of *L.cremoris* ML3

Peptides	Val-Gly Removal Rate by Cell Suspensions ($nmol.h^{-1}.mg^{-1}$)	Val-Gly Removal Rate due to Peptidase Activity ($nmol.h^{-1}.mg^{-1}$)	Net Rate of Val-Gly Uptake by Cells ($nmol.h^{-1}.mg^{-1}$)
1 mM Val-Gly	5000	0	5000
1 mM Val-Gly 4 mM Leu-Gly	1018.9	140.9	878
1 mM Val-Gly 4 mM Glu-Ala	1900	0	1900

Details of the experimental procedure are described in the legend to Table 4.2.6.1

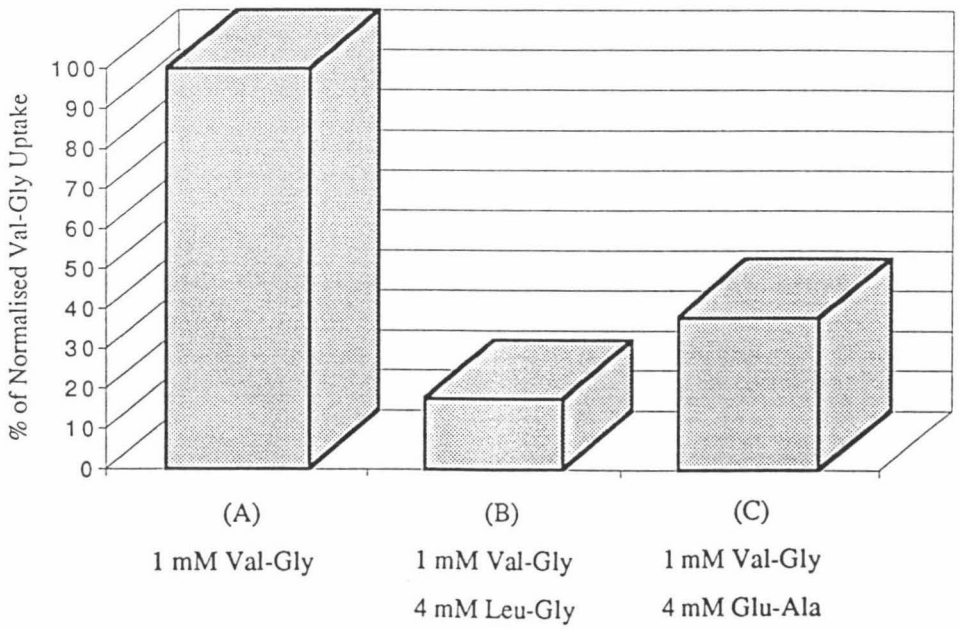


Figure 4.4.4.1 Graphical representation of the data presented in Table 4.4.4.1. Rates of Val-Gly uptake in the presence of Leu-Gly or Glu-Ala have been expressed as a percentage of the control rate.

found that *L. cremoris* AM2 was capable of rapid growth if essential amino acids were supplied as tripeptides rather than dipeptides. From these observations it was proposed that strain AM2 lacks the usual dipeptide transport system. This strain was also proposed to have an oligopeptide system with a high affinity for peptides greater than three amino acid residues and a low affinity for dipeptides, which would account for the slow growth of *L. cremoris* AM2 on dipeptides observed. Such proposals are in variance with the current theory of lactococcal peptide transport (Kunji *et al.*, 1993) which proposes the existence of a common transport system for both dipeptides and tripeptides, and an oligopeptide carrier system with affinity for peptide substrates greater than four residues but not able to transport di- or tripeptides.

Because of the behaviour of *L. cremoris* AM2 in Law's work it was decided to include this strain in the present study.

4.5.1 Growth of *L. cremoris* AM2 in chemically defined medium.

As preparation for a study of the ability of *L. cremoris* AM2 to grow on Val-Gly, Val-Gly_2 or Val-Gly_3 as sole sources of the essential amino acid valine, the ability of this strain to grow in chemically defined medium supplemented with free valine was assessed.
Earlier growth studies with *L. lactis* 1403 had shown that two passages of the culture through CDM were necessary before growth measurements were taken to prevent the carry over of nutrients used in the maintenance of the cultures (Section 2.2.4).

While *L. cremoris* AM2 grew well in the defined medium during these two preparative passages, it failed to grow following a subsequent inoculation into the same medium. This result suggests that some nutrient essential to the growth of strain AM2, while present in the complex broth used to grow the initial inoculum, is absent from the chemically defined medium in which the subsequent growth work was carried out. The failure of *L. cremoris* AM2 to grow in CDM prevented the further investigation of the ability of this strain to use peptides as a valine source for growth.

4.5.2 Uptake of Val-$(Gly)_x$ peptides by *L. cremoris* AM2

Peptide uptake studies on cell suspensions of *L. cremoris* AM2 were able to be carried out using cells harvested from the second passage in chemically defined medium. The net rates of uptake of the synthetic peptides, Val-Gly and Val-Gly_2, are presented in Table 4.5.2.1.

Table 4.5.2.1. Net rates of uptake of Val-$(Gly)_x$ peptides by *L. lactis* subsp. *cremoris* AM_2

Peptide	Peptide Removal Rate by cell suspensions (nmol h^{-1} mg^{-1})	Peptide Removal Rate due to Peptidase Activity (nmol h^{-1} mg^{-1})	Net Rate of Peptide Uptake by Cells (nmol h^{-1} mg^{-1})
Val-Gly	419.3	31.1	388
$Val\text{-}Gly_2$	358.1	0	358

Details for these experiments are given in the legend to 4.1.3.2

The rate of Val-Gly uptake by washed cell suspensions of strain AM_2 is somewhat lower than that seen with the same dipeptide for strains E_8, ML_3 and 920.
Law (1978) also found that the rate of glycyl-(^{14}C)leucine uptake by *L. cremoris* AM_2 was slower than the corresponding rates of uptake by *L. cremoris* E_8 and *L. lactis* 2017, although by a much greater factor than the difference found in the present study.

However, the results are not consistent with Law's observation that *L. cremoris* AM_2 was able to grow strongly on tripeptides as sources of essential amino acids even while growth on dipeptides was minimal. From the large difference in growth rates on dipeptides and tripeptides it would have been expected that peptide uptake rates for tripeptides would be significantly more rapid that those found for dipeptides. Instead the observed rates of uptake of Val-Gly and $Val\text{-}Gly_2$ by washed cell suspensions of *L. cremoris* AM_2 were very comparable.

Due to time constraints it was not possible to pursue this study of peptide utilisation by *L. cremoris* AM_2. The results reported from these preliminary studies do not however indicate that the mechanism of dipeptide and tripeptide uptake in *L. cremoris* AM_2 is significantly different to that of other *cremoris* strains.

4.6 *Lactococcus cremoris* SK11

Of the eight strains of *Lactococcus lactis* subsp *cremoris* that were investigated as part of the extensive study of the ability of lactococci to utilise peptides (Law, 1977;1978), only *L. cremoris* SK11 failed to grow in media where essential amino acids were supplied either as dipeptides or tripeptides.
This inability of *L. cremoris* SK11 to utilise dipeptides or tripeptides is interesting in light of more recent studies with mutants of *L. cremoris* ML3 deficient in peptide transport, which have shown di-/tripeptide uptake to be essential for growth of *L. cremoris ML3* on casein as the sole nitrogen source (Smid, 1989b). This result indicates that one or more essential amino acids must be taken up in the form of a di- or tripeptide. Yet, despite the apparent inability of *L. cremoris* SK11 to utilise dipeptides or tripeptides, this strain demonstates strong growth in both milk and broth media, where amino acids are found predominately as casein peptides.
Other studies in this laboratory (Pritchard *et al.*, 1994) on the peptidase complement of lactococcal strains have shown that the peptidase activities of the SK11 strain are very similar to those of other strains. The inability of strain SK11 to utilise dipeptides and tripeptides cannot therefore be attributed to deficiencies in its internal peptidase complement.
Instead, it appears that this strain is probably incapable of transporting small peptides. This is supported by uptake experiments carried out using radiolabelled dipeptides which showed no significant increase in the internal concentrations of the radiolabelled amino acid (Law, 1978)
It was therefore decided to investigate peptide utilisation in this unusual strain with the model Val-$(Gly)_x$ peptides.

4.6.1 Growth of *L. cremoris* SK11 in chemically defined medium.

Attempts to grow cultures of *L. cremoris* SK11 on CDM, in order to reinvestigate its ability to utilise peptides, yielded the same results as those described earlier for strain AM2. While the SK11 strain grew well during the first two passages in CDM, minimal growth was observed in the third passage in this medium from which growth measurements were taken (Figure 4.6.1.1). This result suggests the absence of a nutrient in CDM which is essential to the sustained growth of both strains AM2 and SK11 (see Section 4.5).
An attempt was made to characterise the possible nature of this essential nutrient requirement. A growth experiment was carried out in which SK11 cells from the second passage in CDM were used to inoculate a third generation of defined

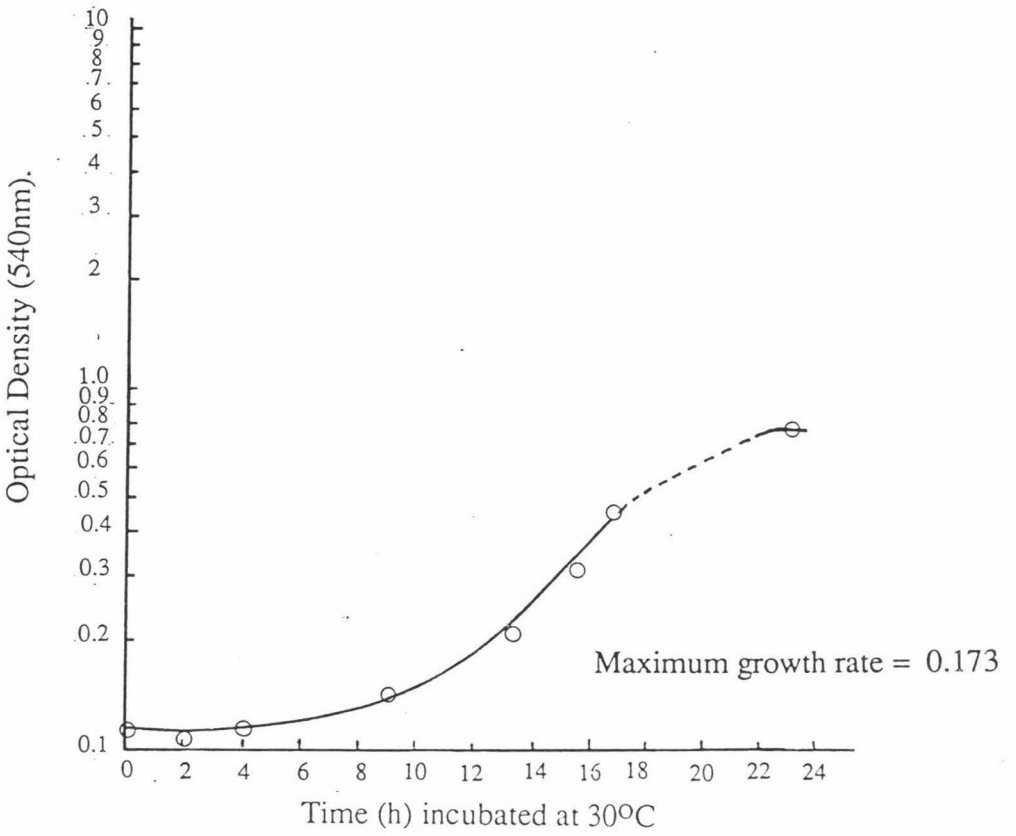


Figure 4.6.1.1 Growth of *L.lactis* subsp. *cremoris* SK_{11} in chemically defined medium, in which all amino acids are supplied in their free state, and containing 1.5% (w/v) lactose. Cells were grown through two passages of this medium before measurements were taken.

Table 4.6.1.1 The effect of substrates upon the growth of *L.cremoris* SK_{11} in chemically defined medium.

Media	OD_{540nm} at 0 h	OD_{540nm} at 24 h	pH at 0 h	pH at 24 h
CDM[a]	0.04	0.56	6.58	6.33
CDM + extra lactose[b]	0.04	0.28	6.57	6.34
CDM + yeast extract[c]	0.04	4.44	6.59	4.48
CDM + beef extract[d]	0.05	4.56	6.58	4.56

[a] CDM consists of the nitrogen component, buffer component, vitamin component and lactose component as described in detail in Section 2.2.1.

[b] extra lactose was added to give a final concentration of 3% (w/v)

[c] yeast extract was added to a final concentration of 10 $g.L^{-1}$, which equates to the concentration found in broth medium.

[d] beef extract was added to a final concentration of 2g $.L^{-1}$, which equates to the concentration found in broth medium.

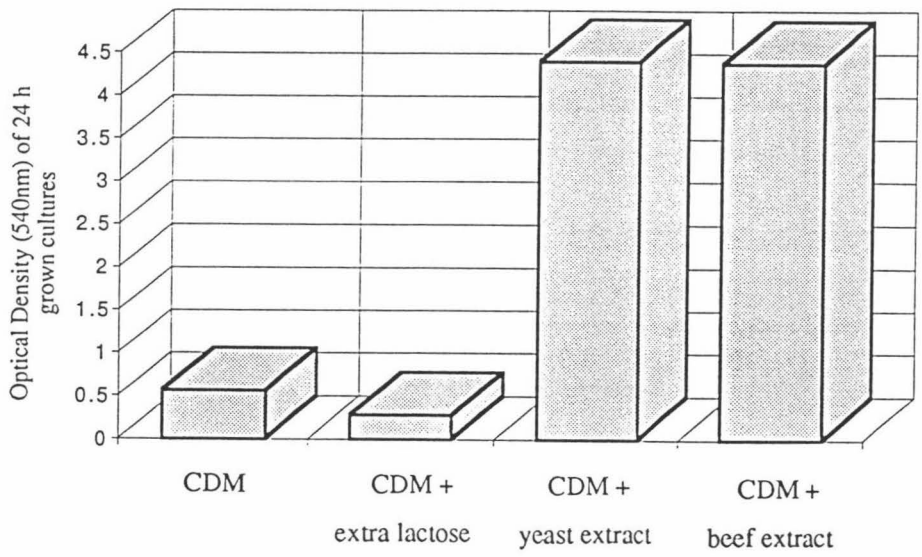


Figure 4.6.1.2 Graphical representation of the data presented in Table 4.6.1.1.

media supplemented with possible growth stimulating nutrients. The results from this study are presented in Table 4.6.1.1 and Figure 4.6.1.2. The addition of extra lactose was found to actually inhibit cell growth in CDM, indicating that carbohydrate exhaustion was not the reason for poor growth of strain SK11 in this medium. In contrast, the addition of either yeast extract or beef extract to chemically defined medium resulted in the cultures growing to high cell densities. These nutrients were chosen as they are important constituents of undefined broth medium (Section 2.1.1) in which *L. cremoris* SK11 grew strongly. Both extracts contain various peptides , vitamins and minerals. The inability of strain SK11 to grow in CDM was probably due to a deficiency in one of these nutrients. It was beyond the scope of this present study to determine precisely what this requirement was.

It was therefore decided to leave the growth work in preference to assessing uptake of the valine-peptides by whole cell suspensions of *L. cremoris* SK11.

4.6.2 Assessment of leakage of intracellular enzymes from *L. cremoris* SK11 cells

As preparation towards studying peptide uptake by *L. cremoris* SK11, the susceptibility of this strain to autolysis was assessed by determining adolase levels in the supernatant of washed cell suspensions (Table 4.6.2.1).

Table 4.6.2.1. Assessment of intracellular adolase leakage from incubating whole cells of *L. lactis* subsp. *cremoris* SK11. Cells were incubated under the same conditions as used in the peptide uptake experiments, except for the omission of peptide.

Time (min)	Aldolase Activity (μmol min^{-1} ml^{-1})		
	(A) Supernatant	(B) French Press Extract	A/B x 100 (%)
0	8.76×10^{-4}	0.169	0.52
30	1.58×10^{-3}	0.214	0.59
60	1.23×10^{-3}	0.267	0.81

(Aldolase levels were determined using the method of Crow *et al.*, 1982)

The results demonstrate that strain $SK11$ is not liable to autolysis and release of intracellular peptidases under the experimental conditions used in the peptide uptake research.

4.6.3 Uptake of Val-$(Gly)_x$ peptides by *L. cremoris* $SK11$.

Peptide uptake studies on stain $SK11$ were carried out using cells harvested after two passages in chemically defined medium.
An attempt was made to determine the rates of uptake of Val-Gly, Val-Gly_2 and Val-Gly_3 by washed cell suspensions of *L. cremoris* $SK11$. Levels of all three peptides in the incubation buffer remained constant throughout the experiments, indicating that none were taken up by the $SK11$ cells. Monitoring of the pH of the cell suspensions showed that lactose was being metabolised into lactic acid.
The absence of peptide uptake was not therefore due to the cell suspensions being metabolically inactive.
While the failure of strain $SK11$ to transport Val-Gly or Val-Gly_2 concurs with earlier findings that *L. cremoris* SK11 is unable to utilise di- or tripeptides (Law, 1977; 1978), the failure to observe transport of all three peptides poses some serious questions about the mechanism by which strain $SK11$ obtains amino acids from its environment. Due to their limited biosynthetic capacities, lactococci must obtain some of the amino acids necessary for growth from the medium in which they grow. However, the results from this current study and from the previous studies of Law (1977; 1978), suggest that *L. cremoris* $SK11$ is unable to transport dipeptides, tripeptides and tetrapeptides.

To assess whether the observed lack of peptide transport might be due to some physiological impairment in the cells after growth through two passages of CDM, Val-Gly_3 uptake was reassessed using broth-grown cells rather than cells grown in CDM. However, no detectable uptake of this oligopeptide was observed. It is possible that the synthetic Val-$(Gly)_x$ peptides used in these studies were not transported because they are not substrates for the transport systems of strain $SK11$. Previous investigation of strain $ML3$ has shown its oligopeptide transport system to have a different substrate specificity to the oligopeptide transport systems of other strains used in this research (Section 4.4.3).
However, earlier work of Law (1977) into the dipeptide utilising capabilities of *L. cremoris* $SK11$, failed to find transport of any of the twelve dipeptides tested. This result suggests that the failure to observe peptide uptake in strain $SK11$ is due to factors other than the specificities of its transport systems.

It was beyond the scope of this current study to investigate further the peculiarities of *L. cremoris* SK_{11} with respect to the mechanism of peptide utilisation and amino acid metabolism.

4.7 Comparative study of the growth of *L. cremoris* strains on chemically defined medium.

The results from the present study of possible lactococcal strain differences, have indicated differences amongst *L. cremoris* strains in their ability to grow on chemically defined medium. Whereas *L. cremoris* E_8 continued to grow rapidly on CDM after two passages in this medium, *L. cremoris* strains SK_{11} and AM_2 failed to grow after two passages in defined medium.

To confirm this observation more extensively, a comparative study of the ability of six *cremoris* strains to grow on CDM was carried out. Cultures of the *cremoris* strains E_8, H_2, HP, AM_1, AM_2, and SK_{11} grown in casein hydrolysate broth (UBM - see Section 2.2.1) were used to inoculate CDM and then grown through further successive passages of CDM. Whereas strains E_8, H_2 and HP were found to be still able to grow to high cell densities in CDM after four passages in this medium, strains AM_1, AM_2 and SK_{11} ceased to grow after the second passage (Figure 4.7.1). Previous studies (Section 2.2.4) have shown that nutrients from the cultures grown in the more complex broth medium used to initiate the growth experiments, can be carried over with the inoculum for two passages in CDM. Thus, only in the third passage in defined medium would components carried over from the broth medium have been exhausted. It is therefore reasonable to assume from the results of this growth study that strains AM_1, AM_2, and SK_{11} are not able to grow in CDM.

The marked difference in growth between these two groups of *L. cremoris* is surprising as DNA hybridisation experiments have shown that the degree of homology between these six strains, relative to *L. cremoris* AM_2, is between 86 and 100 percent (Jarvis *et al.*, 1981).

L. cremoris strains AM_1 and AM_2 were originally isolated in the 1960s as slow-growing starters. Biochemical characterisation showed them to be very similar, except in their resistance to phage. *L. cremoris* SK_{11} is a phage-resistant mutant of *L. cremoris* AM_1, produced by the irradiation of a culture of this latter strain.

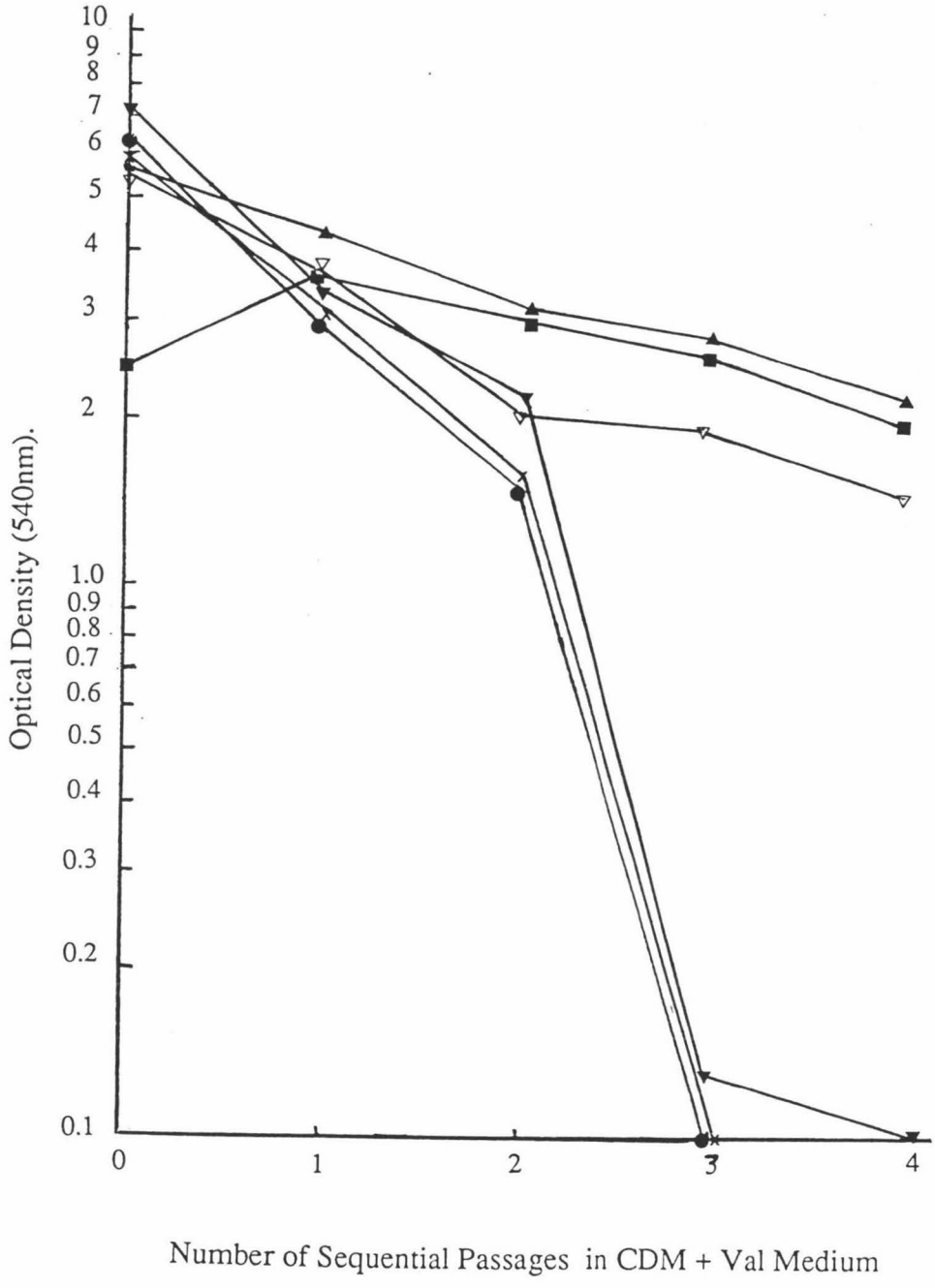


Figure 4.7.1.1 The growth of six *L. lactis* subsp. *cremoris* strains through sequential passages in chemically defined medium containing free valine (CDM + Val). Aliquots (1 ml) of broth grown cells of each strain (generation 0) were used to inoculate the first passage in CDM + Val. After 8 h growth, 1 ml aliquots of these cultures were used to inoculate a new passage of CDM + Val. This procedure was repeated for a maximum of 4 sequential transfers.

L. cremoris H_2	(▲)
L. cremoris E_8	(■)
L. cremoris HP	(▽)
L. cremoris AM_1	(▼)
L. cremoris AM_2	(X)
L. cremoris SK_{11}	(●)

L. cremoris E8, HP and H2 were isolated separately and are probably more diverse in their relationships than strains AM1, AM2 and SK11 (H. Heap, NZDRI - personal communication).

The inability of the *cremoris* strains AM1, AM2 and SK11 to grow on CDM suggests that these three closely related strains have more complex nutritional requirements than *cremoris* strains E8, HP and H2.

Chapter Five: Attempts to use transport mutants in studies on peptide utilisation.

One of the major questions that research on the area of the lactococcal proteolytic system has attempted to answer in recent years, is which of the enzymes and transport systems characterised in these bacteria are essential for growth on milk. Answers to this question have been sought from the study of mutants deficient in either a transport system or an enzyme activity.

These mutants may arise spontaneously at low frequencies and so one approach to their isolation has been the use of synthetic peptides which select for the growth of mutants defective in their ability to use peptides.
The dipeptide L-alanyl-β-chloroalanine (AβClA) has been used to isolate di-/tripeptide transport mutants of *L. lactis* ML3 (Smid, 1991). The selection of a di-/tripeptide transport mutant by the use of this one peptide is possible because lactococci have only one di-/tripeptide system of broad specificity. The potentially toxic nature of this peptide is contained in its β-chloroalanyl residue. This residue is inactive in a peptide form, but once cleaved to the free β-chloroalanine, it acts to irreversibly inhibit a racemase involved in the formation of the cell-wall peptide cross links. While alanine is not essential for the growth of lactococci, growing cells will utilise alanine-containing peptides in preference to synthesising this amino acid themselves. Consequently only cells which are unable to transport AβClA, or lack the enzymes which hydrolyse this peptide, will grow in the presence of AβClA.
Similarly, free β-chloroalanine and AβClA have been used together to isolate *L. lactis* ML3 mutants deficient in both alanine transport and di-/tripeptide transport and which therefore relied solely on oligopeptides as a source of essential amino acids (Kunji *et al.*, 1993).

An alternative approach has been to construct mutants using recombinant DNA techniques. Hagting *et al.* (1994) have created a di-/tripeptide transport mutant of *L. lactis* MG1363 by removing the *DtpT* gene from the cell's genome using homologous recombination. This mutant was unable to grow on CDM in which the essential amino acid glutamate was supplied solely as the dipeptide Ala-Glu, and was resistant to the toxic effects of AβClA. Tynkkynen *et al.* (1993) have isolated and sequenced a DNA fragment from *L. lactis* SSL135 which encoded the Opp proteins involved in oligopeptide transport. Cloning this DNA into an *opp*$^-$ strain of *L. cremoris* ML3 restored the ability of this strain to utilise peptides

containing four or more amino acid residues.

Extensive studies of these transport mutants have shown that both the di-/tripeptide transport system and the oligopeptide transport system are essential for the growth of lactococci on milk.

The dipeptide AβClA was synthesised, characterised and used in an attempt to isolate mutants unable to utilise dipeptides. The inability to use dipeptides could be due to deficiency in either transport or dipeptidase activity.
An objective of this current study when initially planned was to isolate a spontaneous dipeptidase mutant of *L. cremoris* E8 using AβClA. However, recent gene disruption experiments have shown that aminopeptidase N (Mayo *et al.*, 1993), endopeptidase O (van Alen-Boerrigter *et al.*, 1994), and X-prolyl-dipeptidyl-aminopeptidase (Mierau *et al.*, 1993) activities are not essential to growth on casein. If lactococcal dipeptidase activity is also not essential for growth then it would not be possible to isolate a dipeptidase mutant with the techniques employed in this study.
However, use of a di-/tripeptide transport mutant would provide evidence confirming the validity of the uptake procedure used in the present study as a measure of activity of the di-/tripeptide transport system. Such a mutant would also be useful to establish that uptake of oligopeptides was due to a separate transport system as indicated by the work of Smid (1991) and Kunji *et al.* (1993).
It was decided to use the synthesised AβClA to attempt to isolate a di-/tripeptide transport mutant of *L. cremoris* E8. The characterisation of the synthetic peptide and its use in attempts to isolate mutants are described in Sections 5.1 and 5.2 respectively.

Results are also presented from peptide utilisation studies with an oligopeptide transport mutant supplied by Dr L. McKay, University of Minnesota (Section 5.3).

5.1 Characterisation of the product of L-alanyl-β-chloroalanine synthesis.

The toxic dipeptide L-alanyl-β-chloroalanine (AβClA) was synthesised using solution phase chemistry as detailed in Section 2.2.11. An HPLC profile of the crude product from this synthesis is shown in Figure 5.1.1. Samples of each of the two major peaks in this profile were sent for molecular weight determination using liquid SIMS. The second of these peaks, (b), had a mass of 195 which corresponded to the expected mass of the MH^+ ion of AβClA of 194.7 (Figure 5.1.2). Importantly this profile lacked a peak at mass 123.6, the expected mass for

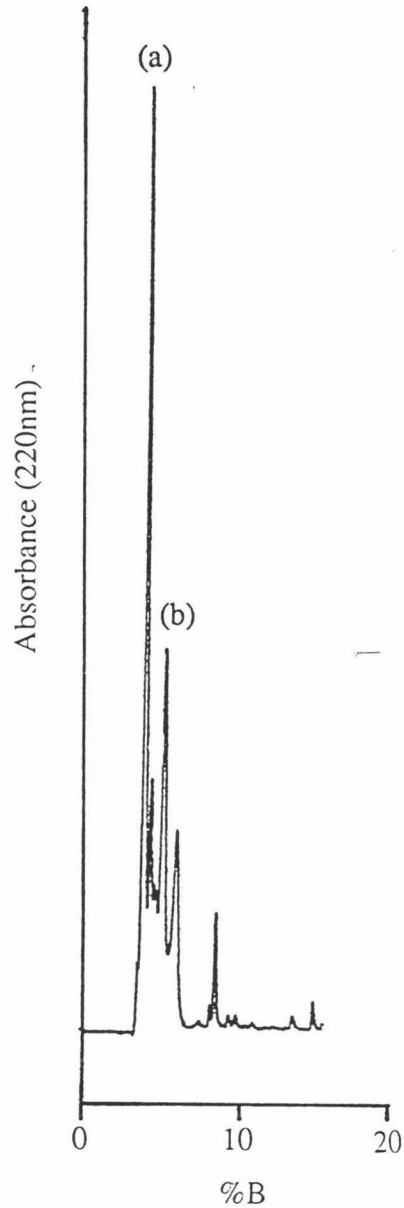


Figure 5.1.1 Reverse phase HPLC profile of the crude peptide product resulting from the synthesis of L-alanyl-β-chloroalanine. (a) and (b) mark the peaks that were sent for mass determination. Solvent A was 0.1 % TFA in water, and solvent B was 0.08 % TFA in acetonitrile. The products were eluted with a linear gradient of 0 - 15 % B over 15 min.

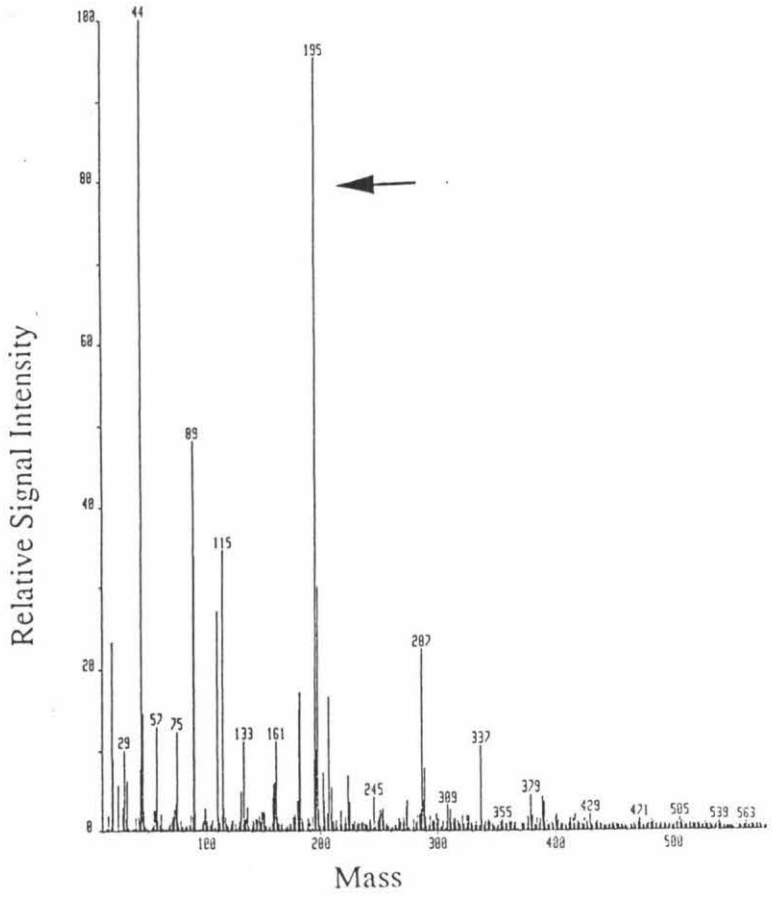


Figure 5.1.2 Mass spectrum of peak (b) collected from an HPLC separation of the crude product from L-alanyl-β-chloroalanine synthesis (see Figure 5.1.1). The arrow marks the mass corresponding to the expected mass of the MH^+ ion of L-alanyl-β-chloroalanine.

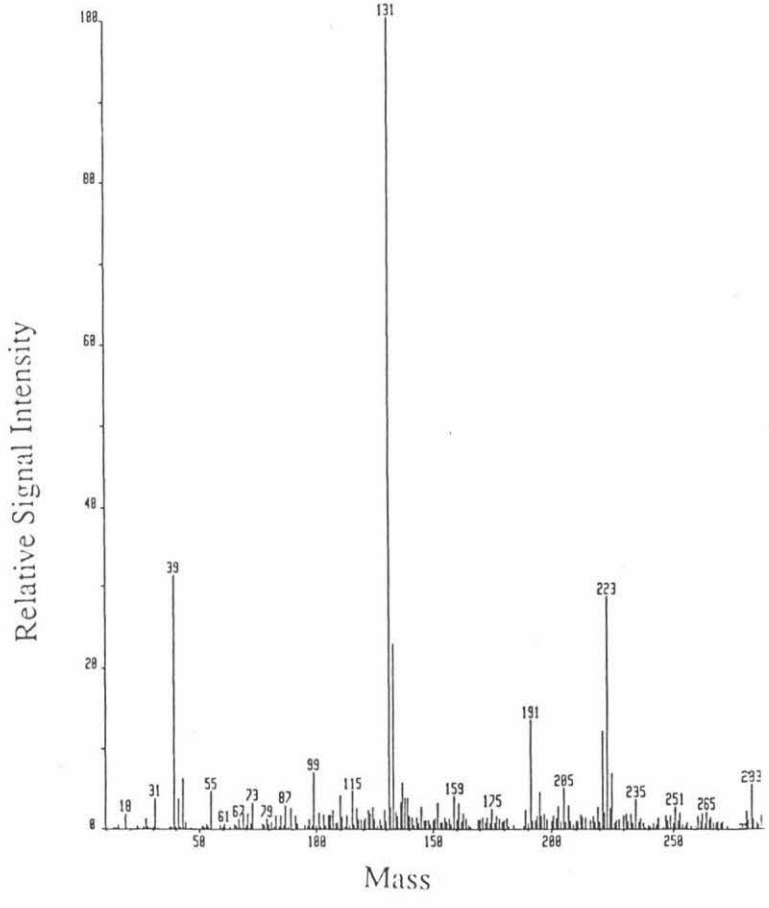


Figure 5.2.1 Mass spectrum of purified L-alanyl-β-chloroalanine collected from a subsequent HPLC separation of the crude product.

free β-chloroalanine. Because this is the toxic component of AβClA, its presence in the synthesised product would result in the selection of a mutant different to that which was desired.

Due to the small amount of crude peptide available it was decide not to attempt a large scale purification of the toxic dipeptide by preparative HPLC. Instead small amounts of AβClA were purified by collecting the peak of interest from repeated analytical HPLC separations of the crude product.

5.2 The attempted isolation of dipeptide-transport mutants of *L. cremoris* E8 using L-alanyl-β-chloroalanine.

Attempts were made using both the crude crystals and the HPLC purified AβClA to isolate di-/tripeptide transport mutants of *L. cremoris* E8 using the procedure described in detail in Section 2.2.11. Zones of inhibition were seen quite clearly around these sources of toxic peptide. No corresponding zone was seen around the TFA control. Four separate colonies growing within the inhibition zones were isolated , grown initially on CDM and then tested for growth in defined media in which valine had been substituted for by Val-Gly or Val-Gly_2. All of the four colonies isolated grew well on both these media. Their apparent resistance to the effects of AβClA cannot therefore be attributed to a deficiency in their ability to transport dipeptides.

It was possible that the absence of AβClA from the media used for the test plating of suspected mutants allowed the growth of cells which had spontaneously reverted to the wild-type phenotype.

Further attempts to isolate a di-/tripeptide transport mutant were made in which AβClA was added to the test media to maintain the selection pressure. However again all isolates from the inhibition zones grew on the test-plates containing Val-Gly or Val-Gly_2 as sole sources of valine.

This continued failure to isolate a transport mutant was surprising as the procedure used to select for such mutants was very similar to that used in the original study (Smid *et al.*, 1989).

In an attempt to identify reasons for this failure, a further sample of the HPLC-purified AβClA was sent again for molecular weight determination. The results of this analysis (Figure 5.2.1) showed a major peak at a mass of 131, but none at the expected mass of 195. This result suggests that the material collected from the later HPLC separation and used in the mutant isolation experiments, did not contain the toxic peptide any longer, which would account for the failure to isolate mutants. No explanation can be offered to account for the discrepancy between the analytical

data for the two different preparations of the peptide. Due to time constraints further attempts to synthesise authentic alanyl-β-chloroalanine were not pursued.

5.3 Peptide uptake by the oligopeptide transport mutant, *Lactococcus lactis* subsp. *lactis* KG301

The ability of lactococci to hydrolyse and grow on milk caseins has been described as the proteinase positive (Prt^+) phenotype. Recent genetic studies have indicated that functional genes encoding proteinase activity (*prtP*), di-/tripeptide transport (*dtpT*), and oligopeptide transport (*opp*) are all required for the Prt^+ phenotype. While the *dtpT* gene appears to have an exclusively chromosomal location, and the *prtP* gene has a plasmid location, the *opp* gene cluster can be found on either the chromosome or a plasmid.
A Prt^- mutant deficient in its ability to transport oligopeptides was obtained from Dr L. McKay of the University of Minnesota at a very late stage of this present investigation. This mutant was derived from *L. lactis* LM2301 (Walsh and McKay, 1981), a Prt^- strain, which lacks the genetic loci for lactose utilisation, the cell wall-associated proteinase and the oligopeptide transport system. The plasmid pJK550, which contains the *lac* and *prtP* genes was introduced into LM2301 to produce the *lac*$^+$ *prtP*$^+$ *opp*$^-$ strain, *L. lactis* KG301.
Studies in chemically defined media have shown that *L. lactis* KG301 is unable to utilise a pentapeptide as the sole source of the essential amino acid leucine, and is unable to grow on casein as the sole amino acid source (Yu, 1994).

To provide further evidence supporting the validity of the procedure used throughout the present study as a measure of oligopeptide transport, peptide uptake experiments using the peptides Val-Gly_2 and Val-Gly_3 were carried out with cell suspensions of the strain KG301. Whereas the tripeptide Val-Gly_2 was utilised by *L. lactis* KG301 , the tetrapeptide Val-Gly_3 was not (Table 5.3.1). As discussed above, *L lactis* KG301 possesses all the components of the lactococcal proteolytic pathway, except the oligopeptide transport system. The failure to observe any decrease in supernatant Val-Gly_3 levels therefore indicates that peptide disappearance from the medium reflects the activity of the oligopeptide transport system.

The ability of *L. lactis* KG301 to utilise the β-casein oligopeptide QEPVLGPVRGPFPIIV was also assessed. No detectable change occurred in the supernatant levels of this hexadecapeptide. This result suggests that the slow rate of uptake observed when this peptide was incubated with *L. cremoris* E8 (Section

4.2.8) was due to its active transport as an intact peptide by the oligopeptide transport system.

Table 5.3.1. Net rates of uptake of Val-$(Gly)_x$ peptides by *L.lactis* subsp. *lactis* KG301

Peptide	Peptide Removal Rate by cell suspensions (nmol h^{-1} mg^{-1})	Peptide Removal Rate due to Peptidase Activity (nmol h^{-1} mg^{-1})	Net Rate of Peptide Uptake by Cells (nmol h^{-1} mg^{-1})
$Val\text{-}Gly_2$	1386.9	0	1387
$Val\text{-}Gly_3$	0	0	0

Details for these experiments are given in the legend to 4.1.3.2

Chapter Six: Discussion

6.1 Peptide metabolism and the lactococcal proteolytic pathway.

The growth of lactococci in milk is possible only because they possess a complex proteolytic system which enables these nutritionally fastidious group of bacteria to utilise milk caseins as sources of essential amino acids.

The three major components of this proteolytic system are the proteinases, the peptidases and the transport systems. Much information is now available about the genetic determinants , molecular structure, specificities and modes of activity of the cell wall-associated proteinases (Visser *et al.*, 1986; Exterkate and de Veer, 1989; Kok, 1990, 1991; Reid *et al.*, 1991a, 1991b, 1994), the intracellular peptidases (Pritchard and Coolbear, 1993; Kok *et al.*, 1994) and the cell membrane-bound amino acid and peptide transport systems (Konings *et al.*, 1989; Smid *et al.*, 1989a, 1989b; Kunji *et al.*, 1993; Tynkkynen *et al.*, 1993; and Hagting *et al.*, 1994).

Questions remain, however, about how these separate components interact *in vivo* to enable lactococci to metabolise milk-caseins. The most important of these questions is concerned with the processes of peptide metabolism; that is, the mechanisms by which cells transfer the oligopeptides products produced by casein hydrolysis into an intracellular pool of useable amino acids (see Figure 6.1.1). The role of the cell wall-associated proteinase in cleaving milk caseins into large oligopeptides at the proximity of the cell wall is now well established. Similarly, the activities and possible roles of the internal peptidase complement in breaking the later products of digestion is also well understood. However it has not been established how the early products of proteinase activity enter the cell. Two models have been proposed from the research available to date. In the first of these, the large oligopeptides produced by the proteinase are degraded by extracellular peptidases into small peptides and free amino acids for transport into the cell. As yet though, no peptidases with an unequivocally extracellular location have been located. The alternative model proposes that the large oligopeptides are transported without prior hydrolysis. While such a model alleviates the need for extracellular peptidase activity, it is not unequivocally supported by current knowledge available on the transport systems, especially the oligopeptide transport system. A central aim of this present study was to try and define the route(s) by which the products of casein hydrolysis cross the cell membrane. This answer was sought by investigating more fully the *in vivo* activity of the lactococcal oligopeptide transport system.

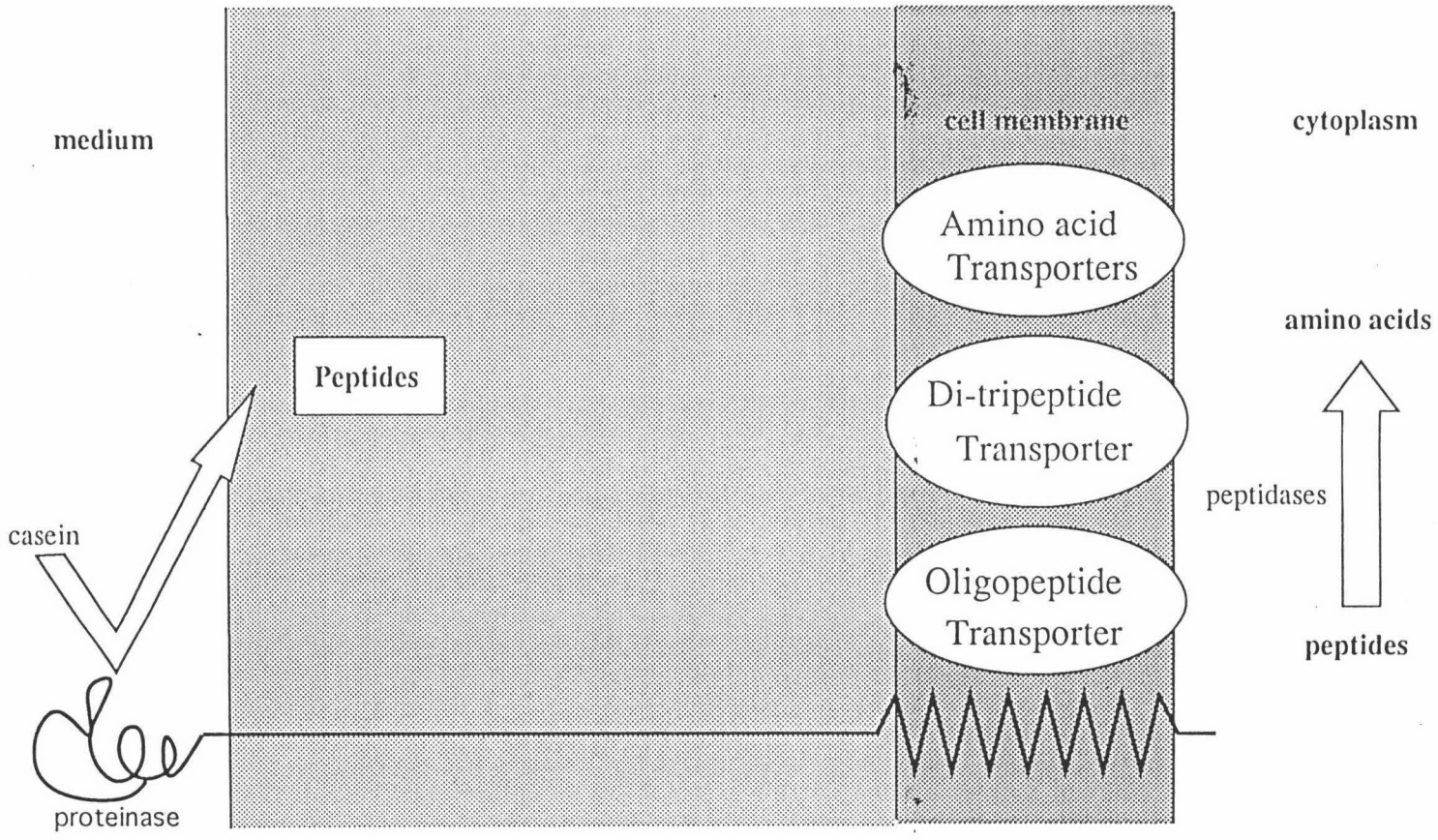


Figure 6.1.1. Diagrammatic representation of the processes and components involved in casein utilisation, indicating those which have been well characterised.

A second question of concern in this study was the validity of making generalisations about the mechanisms of peptide metabolism from the results of the study of a single lactococcal strain. Most of the knowledge on which current models for the utilisation of casein peptides are based comes from the extensive study of the ML3 strain of *L. cremoris*, and its related mutants (Smid, 1991; Kunji *et al.*, 1993: Tynkkynen *et al.*, 1993; and Hagting *et al.*, 1994). However earlier research (Law, 1977; 1978) has proposed significant differences between *L. lactis* and *L. cremoris* strains with respect to the mechanisms by which they utilise di- and tripeptides. A further aim of this research was therefore to reinvestigate possible strain differences in peptide utilisation, and assess the soundness of using strain ML3 as a paradigm of lactococcal transport.

A third important question concerning peptide metabolism which this study attempted to address was which of the various lactococcal transport systems and peptidases characterised are *essential* components of the proteolytic pathway. The isolation of mutants defective in either the di-/tripeptide transport system (Smid *et al.*, 1989b) , or the oligopeptide transport system (Tynkkynen *et al.*, 1993; Yu, 1994) have shown that both are essential for casein utilisation. An attempt was made to isolate a mutant of *L. cremoris* E8 which may have been defective in its ability to hydrolyse dipeptides and/or to transport dipeptides. The isolation of a dipeptidase mutant would enable this study to assess the necessity of this enzyme for the supply of essential amino acids for growth. Alternatively, the isolation of a dipeptide transport mutant would be useful in establishing not only that di-/tripeptide transport was an essential process, but also that uptake of oligopeptides was due to a separate transport system in a strain other than *L. cremoris* ML3.

Finally, while the factors involved in the regulation of proteinase expression and activity have been well studied, much less is known about possible regulators of the peptidase and transport components of peptide metabolism. The results of earlier research have yielded conflicting evidence with respect to the question as to whether peptidase activities are influenced by the type of nitrogen source upon which the cells are growing (Law, 1977; van Boven and Konings, 1988). No information is available on whether the expression of the peptide transport systems is regulated or constitutive. An attempt was made in this study to determine whether the expression and/or activities of these components are nutritionally regulated.

6.2 The validity of using whole-cell peptide uptake as a measure of peptide transport

The major procedure employed in this study to examine the questions discussed in the previous section, was the determination of rates of whole-cell peptide uptake. These rates can be assessed for cell suspensions, either by measuring the accumulation of a peptide within the cells, or by following the disappearance of that peptide from the supernatant of the cell suspension.

While the first of these procedures for assessing peptide uptake is preferable, it is complicated by the fact that lactococcal cells hydrolyse peptides into their constituent amino acids immediately they enter the cell. Early studies of whole-cell peptide transport (Law, 1977; Rice *et al.*, 1978) have used peptide substrates containing a radiolabelled residue. The rate at which this label accumulated within the cytoplasm has been used as a measure of peptide transport. However, there are two inherent problems in using such a method as a measure of transport. Firstly, resting cells actively excrete amino acids produced by the internal hydrolysis of peptides (van Boven and Konings, 1989), and the rate of excretion differs between amino acids. As a consequence, amino acids will differ in the rate at which they accumulate within cells. Any assessment of peptide uptake based on the accumulation of a single amino acid residue from that peptide will be influenced by the particular amino acid chosen. In all cases however, the rates determined are likely to be an underestimate of the actual rate of uptake.
Secondly, while the appearance of a radiolabel within a cell provides direct evidence that the amino acid is being transported, it does not provide information about the form in which that amino acid was transported, *i.e.*, whether it was as the original peptide, a smaller peptide derived from the original substrate, or as the free amino acid. Researchers using this technique to assess peptide transport have relied upon indirect evidence, such as the inability of unlabelled amino acids to inhibit the accumulation of the labelled residue, the dependence of this accumulation upon a viable energy source, and the suppression of accumulation by specific inhibitors of peptide transport, to demonstrate that the labelled residue entered the cell as the original peptide substrate.

A recent study of di-/tripeptide transport has circumvented these problems by cloning the gene for the lactococcal di-/tripeptide transport system into a strain of *E. coli* which did not possess its own transport system , and which also was unable to degrade the dipeptide Ala-(^{14}C)Glu (Hagting *et al.*, 1994). For the first time these researchers were able to incontestably demonstrate that the increase in the intracellular concentration of the radiolabelled amino residue was due to transport of the intact peptide.

The alternative procedure of assessing whole-cell peptide transport by measuring the disappearance of a peptide from the supernatant of a cell suspension, was the method of approach chosen for this current study. This procedure has the advantage of not having to use radioactive peptides which involve particular problems in both their synthesis and use. However, a major disadvantage with this approach was the absence of direct evidence that the peptide was being taken up as such by the cells as this system relies on measuring the disappearance of a substrate outside the cells, rather than measuring its accumulation in the cytoplasm.

It is possible that the rates of decline in supernatant peptide levels measured with cell suspensions were due to the hydrolysis of those peptides by extracellular peptidases or cell wall-associated peptidases rather than as a result of peptide transport. However the results obtained support this being the case for only one of the strains investigated. An important feature of the experimental design, was the assessment of peptidase levels for a timed interval once the cells were removed from the incubation system. Except for *L. lactis* 1403, peptide levels remained constant during this subsequent incubation, which would not have been the case if the disappearance of peptide was a consequence of extracellular hydrolysis.

The use of capillary electrophoresis (CE) to determine peptide levels in the samples taken from the incubation systems also provided evidence that the decline in supernatant peptide levels was due to transport rather than the activity of cell wall-associated peptidases. CE is a highly sensitive analytical technique which is able to resolve peptides differing in only one amino acid , even when that difference is as small as a single methyl group; for example, Val-Gly and Leu-Gly. Hydrolysis of a peptide , either by extracellularly located peptidases or by cell wall-associated peptidases, can be detected by looking for peaks in the CE profiles that correspond to possible cleavage products of the original peptide substrate. Such a peak was observed when assessing Val-Gly_2 uptake by *L. lactis* 1403. Extracellular cleavage of this peptide released a diglycine peptide which appeared in the CE profiles of the timed samples. Assessment of adolase levels in a cell suspension of this strain implicated leaked internal peptidases as being responsible for this cleavage. The failure to observe a corresponding peak in the CE profiles of timed samples taken to determine Val-Gly_2 utilisation by other strains supports the conclusion that this peptide entered these strains intact. Furthermore, no addition peaks appeared in the CE profiles produced when larger Val-$(Gly)_x$ peptides, or β-casein oligopeptides were used as substrates in the whole-cell uptake systems suggesting that their disappearance from the supernatant was also due to transport rather than hydrolysis. The results from the dipeptide competition experiments also support the validity of using dipeptide disappearance as a measure of the rate of transport of the intact peptide.

It is also possible that the rates of peptide utilisation measured were in part attributable to some non-specific interaction with the cells in the incubation system, such as absorption to the cell walls. It was therefore important to demonstrate that peptide utilisation was both an active and a specific process, as would be expected if the process of peptide utilisation was due to uptake by a peptide transport system.
The omission of glucose from the incubation systems prevented any decline in the supernatant levels of dipeptide, tripeptide and oligopeptide substrates. Furthermore the addition of the proton motive force inhibitor carbonyl cyanide tri-chlorophenyl hydrazone (CCCP) completely suppressed the uptake of Val-Gly_2 by cell suspensions.

Finally, the failure of the oligopeptide transport mutant *L. lactis* KG301 to utilise either Val-Gly_3, or the β-casein peptide QEPVLGPVRGPFPIIV, supports the validity of using oligopeptide utilisation as a measure of actual transport by the cytoplasmic membrane-bound oligopeptide transport system. This result is particularly important since an alternative mechanism to account for part or all of the utilisation of oligopeptides by whole cell suspensions could be a tightly coupled cell wall (or membrane) peptidase-di-/tripeptide (or amino acid) uptake system. If such a tightly coupled system was operating, small peptide products of the peptidase step would not be detectable even by the sensitive technique of capillary electrophoresis. However, if this were the pathway for oligopeptide transport it would still function in a mutant specifically deficient in the oligopeptide transport system.

Throughout the presentation of the results obtained in this study, the expressions "peptide utilisation" and "peptide uptake" have been used in preference to "peptide transport" because of potential limitations in the methodology used. However from the collective results of the experiments discussed above, it seems valid to conclude that the procedure used in this study is an accurate measure of whole cell peptide transport. Therefore in future discussion of the results , "peptide transport " will be used in preference to the less precise terms of "peptide utilisation" and "peptide uptake".

6.3 Lactococcal oligopeptide transport and its possible role in the utilisation of β-casein oligopeptides

As discussed in Section 6.1, two models have been proposed concerning the mechanism by which the initial oligopeptide products, produced from the cleavage of milk caseins by the cell wall-associated proteinase, enter the cell. One of these proposes extracellular hydrolysis of the oligopeptides to di-or tripeptides or free amino acids by peptidases located outside the cell membrane. The evidence used cited in favour of this model relies to a large extent on our current knowledge of the lactococcal oligopeptide

transport system, since unequivocal evidence for extracellular peptidases is lacking. The average size of the oligopeptides produced by cleavage of β-casein is 11 - 12 residues. Previous studies, however, have suggested that the upper size limit of oligopeptides capable of being translocated by this system is 5 - 6 residues (Law, 1978; Rice *et al.*, 1978; Smid, 1991). Consequently, the β-casein oligopeptides would have to be hydrolysed into smaller peptides before they could be taken up. Furthermore, studies of a di-/tripeptide transport mutant of *L. lactis* ML_3 (Kunji *et al.*, 1993) have suggested that the activity of the oligopeptide transport system *in vivo* is much lower than that of the di-/tripeptide transport system.
For these reasons, it has generally been accepted that the oligopeptide transport system does not play a significant role in the lactococcal proteolytic pathway other than that of transporting peptides containing essential amino acids that are not available to the cell as either dipeptides or tripeptides.

The results from this current study have suggested that the functionality of this transport system may not be as limited as currently believed. Peptide transport experiments with the *L. cremoris* strain E8 using the Val-$(Gly)_X$ peptides have shown conclusively that the oligopeptide transport system of this strain is capable of translocating peptides with at least 8 residues. This work was supported by growth studies which showed that *L.cremoris* E8 can utilise oligopeptides containing up to 8 residues as sources of the essential amino acid valine, and which provided indirect evidence that this was a result of transport of the intact peptide.
This result is in agreement with that from a more recent study of the possible upper size limit of the oligopeptide transport system (Tynkkynen *et al.*, 1993), and is closer to the average size of the oligopeptides initially produced from the hydrolysis of casein.
The preliminary results from the transport studies carried out with the β-casein oligopeptides have provided evidence that the upper size limit of peptides capable of being transported by the oligopeptide transport system may be considerably higher, since the 16 residue oligopeptide was apparently taken up without detectable hydrolysis to smaller peptides.

However, the apparent ability of the oligopeptide transport system to transport peptides as large as hexadecapeptides is only of physiological significance if the rates of uptake of these peptides is rapid enough to account for the rates of cell growth seen in milk. The results from the transport studies using *L.cremoris* E8 cell suspensions and the valine-peptides have shown that oligopeptides are transported at rates equal to those for the transport of di- and tripeptides. Little information is available about the rates of transport of the larger β-casein peptides although it appears that this rate is comparatively much slower.

It therefore appears that, at least for *L. cremoris* E8, the oligopeptide transport system could play a significant role in peptide metabolism, as proposed in Figure 6.3.1. The main feature of this alternative model is the ability of cells to utilise the large oligopeptide products produced from proteolytic cleavage of milk caseins without the need for hydrolysis into smaller peptides and amino acids. Such a model would account for the failure to date to isolate peptidases with extracellular locations.

The results from this current study also suggest that the lactococci are quite distinct with respect to their mechanism of peptide transport from *Escherichia coli* and *Salmonella typhimurium*. The peptide transport systems of these two bacteria are extremely well characterised, and the structure of the oligopeptide transport system of *Lactococcus lactis* was modelled upon that of *S. typhimurium*. (Tynkkynen *et al.*, 1993). However the oligopeptide permease system of *S. typhimurium*. will only transport peptides containing between 2 and 5 residues. Extracellular hydrolysis of peptides by peptidases therefore plays an important role in the proteolytic pathway of these bacteria. In contrast the larger upper size limit of the oligopeptide transport system of *Lactococcus lactis* may enable these bacteria to utilise peptides directly from their environment without the need for extensive extracellular hydrolysis.

6.4 Strain differences in the mechanisms of peptide utilisation.

The utilisation of peptides from the environment by lactococci involves the activity of both transport systems and peptidases. A major emphasis of this research project was a comparative study of peptide utilisation by *L. cremoris* and *L. lactis* strains. This work involved growth studies on a chemically defined medium, assessments of intracellular peptidase activities and peptide transport studies using a model peptide series. The results obtained from these studies were presented and discussed in detail on a strain by strain basis in Chapter Four. The intention of this section is to draw together this work to address two fundamental questions about lactococcal peptide metabolism, namely, are there significant differences in the mechanisms by which lactococci utilise casein-derived oligopeptides, and if so, to what extent are the generalisations made about lactococcal peptide metabolism from the study of *L. cremoris* ML3 valid for other strains ?

A summary of the data obtained when strains 1403, 920, E8, ML3, and SK11 were grown in chemically defined medium is presented in Figure 6.4.1 and Table 6.4.1. Significant differences are apparent between these strains in both their maximum rates of growth and in the final OD_{540nm} reached. One strain , *L. cremoris* SK11, grew minimally in this medium, suggesting that the medium lacks adequate quantities of

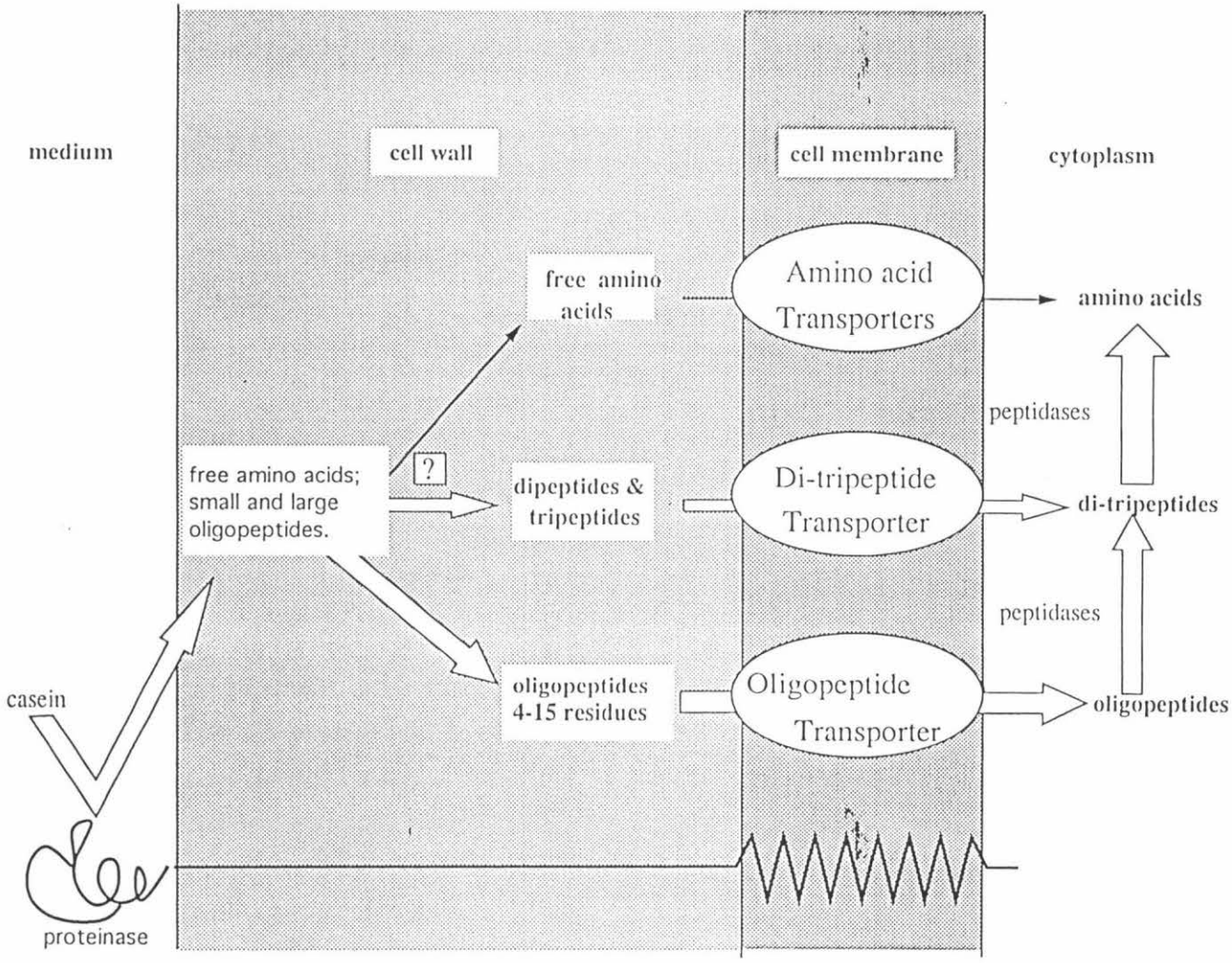


Figure 6.3.1. A proposed model for the mechanism of utilisation of milk caseins by *L. lactis* subsp.*cremoris* E8

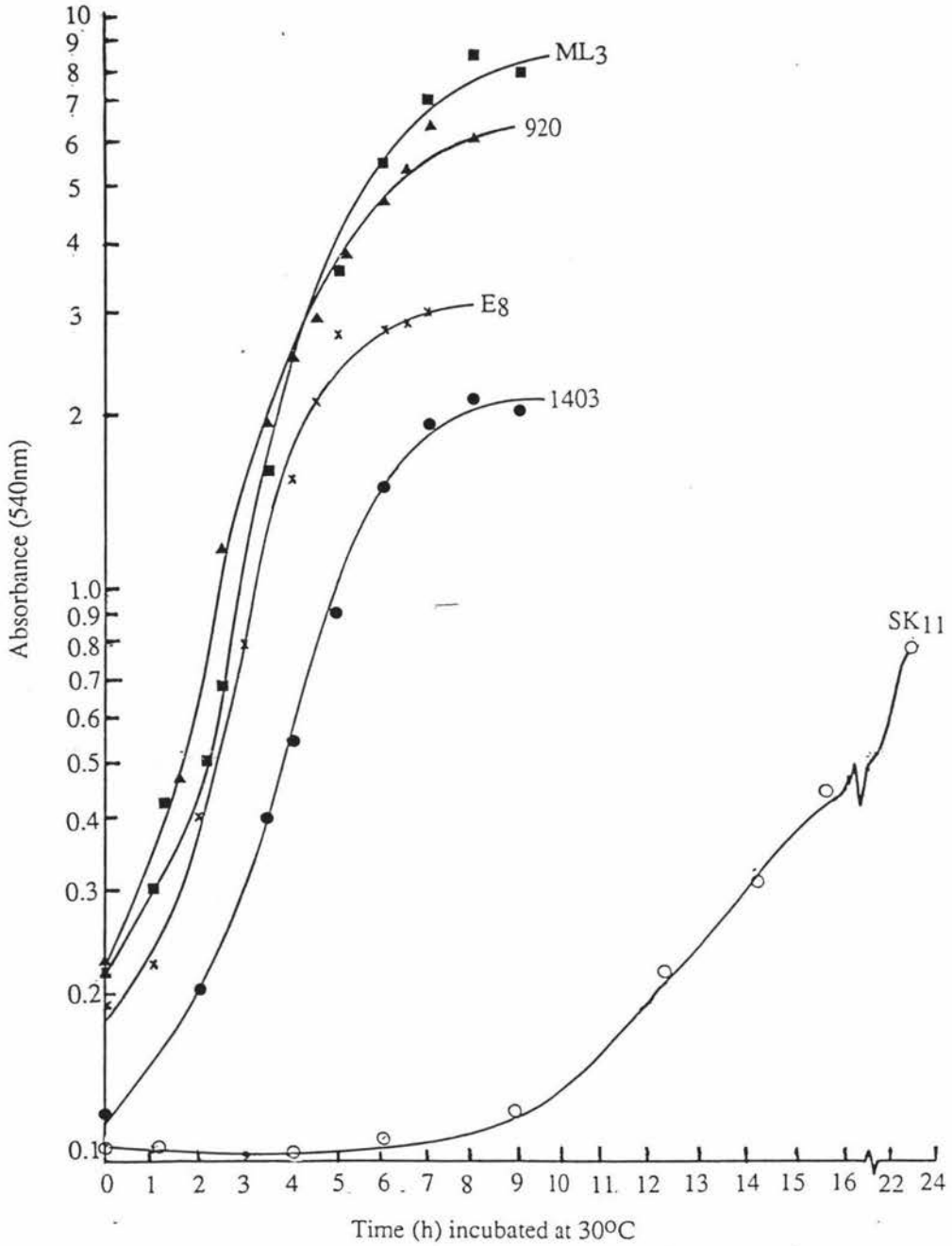


Figure 6.4.1 The growth of *L. lactis* subsp.*lactis* and *L. lactis* subsp.*cremoris* strains on chemically defined medium in which all amino acids are supplied in their free state, and containing 1.5 % (w/v) lactose.

L. lactis subsp *lactis* 1403	(●)
L.lactis subsp *lactis* 920	(▲)
L. lactis subsp *cremoris* SK_{11}	(○)
L. lactis subsp *cremoris* E_8	(X)
L. lactis subsp *cremoris* ML_3	(■)

Cells were grown through two passages of this medium before measurements were taken.

Table 6.4.1 Specific growth rates of *L.lactis* subsp.*lactis* and *L.lactis*.subsp *cremoris* strains on chemically defined medium.

Strain	Maximum Growth Rate in CDM
L. lactis 1403	0.630
L. lactis 920	0.693
L. cremoris SK_{11}	0.173
L. cremoris E_8	0.770
L. cremoris ML_3	0.924

[a] determined over the logarithmic phase of growth using the expression $\frac{\log_e 2}{\text{doubling time}}$

some nutrient essential for the growth of this strain. The other four strains , 1403, 920, E8 and 1403, all grew well in the chemically defined medium used in the present study, although these were considerable differences in the final growth yield attained. An investigation of the possible reasons for the differences in growth yield of the ML3 strain (final OD_{540nm} of 8.5) and the 1403 strain (final OD_{540nm} of 2.1) revealed that the growth yield of ML3 was limited by the carbohydrate supply (lactose exhausted at the end of the log phase), while the growth of 1403 was limited by some other essential nutrient (residual lactose present at the end of the log phase) [data not shown]. Amino acid analyses of the medium in which the cells were growing showed that the entry of a culture of 1403 into the stationary phase was not due to the exhaustion of one or more essential amino acids.

Comparison of the peptidase activities from French Press extracts of cultures of the strains 1403, 920, E8 and ML3 grown previously in CDM, show only relatively small differences between the strains (Table 6.4.2). Further replication of the assays would be necessary to establish whether such differences as were found (e.g. in the prolidase and lysine aminopeptidase activities) were reproducible, and significant. The present result is consistent with the observations of van Boven and Konings. (1988), who found that intracellular peptidase activities in *L. cremoris* strains E8 and Wg2 were comparable. The results from this study support the proposal made by these authors that strain differences in peptide utilisation are not a result of differences in internal peptidase activities.

However, differences were found between strains in their ability to transport the Val-$(Gly)_x$ peptides (Table 6.4.3). From the results of the whole-cell peptide uptake studies these strains can be divided into three categories: those that are unable to transport peptides; those where some extracellular hydrolysis occurs, releasing free amino acids which may be taken up as such ; and finally those that transport peptides as such without any prior hydrolysis.

The first of these three categories is represented by *L. cremoris* SK11. This study failed to detect transport of either dipeptides, tripeptides or oligopeptides by this strain. These results are enigmatic as *L. cremoris* SK11 demonstrates strong growth in milk which requires utilisation of casein oligopeptides.

The second category is represented by the single strain, *L. lactis* 1403. With this strain, considerable leakage of peptidase activity occurs, probably due to the autolysis of a proportion of the cell population. This results in hydrolysis of some of the peptide, so that part or all of the amino acid uptake may be in the form of free amino acids rather

Table 6.4.2 Comparison of the peptidase activities of French Press extracts of *L. lactis* and *L. cremoris* strains grown in chemically defined medium. Peptidase activities are expressed as μmol min^{-1} mg^{-1} protein.

Strain	Dipeptidase Ala-Ala	Tripeptidase Leu-Gly_2	Prolidase Leu-Pro	Pro/imino-peptidase Pro-Ala	Lysineamino peptidase Lys-*p*NA	X-prolyl dipeptidyl aminopeptidase Gly-Pro-AMC
1403	3.14	0.432	0.321	0.016	0.124	0.228
E_8	1.71	0.483	0.128	0.008	0.126	0.261
ML_3	2.12	0.467	0.205	0.016	0.282	0.197
920	2.98	0.642	0.383	0.012	0.089	0.160

Table 6.4.3 Comparison of the net rates of transport of Val-$(Gly)_x$ peptides by *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* strains.

Peptide	Net Rate of Peptide Transport (nmol h^{-1} mg^{-1} dwc).					
	L. lactis strain		*L. cremoris* strain			
	1403	920	E_8	ML_3	SK_{11}	AM_2
Val-Gly	0*	580	761	3702	0	388
Val-Gly_2	317*	1453	872	1047	0	358
Val-Gly_3	1149	915	731	0	0	ND
Val-Gly_4	ND	677	866	ND	ND	ND
Val-Gly_7	ND	451	777	ND	ND	ND

* These rates represent minimal estimates since they are obtained by subtraction of peptidase hydrolysis rates measured at the end of the experiment (see text for discussion).

than as peptides. Since peptidase activity was measured at the end of the 2 h period used to assess whole cell peptide utilisation rates, the rate of peptide utilisation calculated as due to extracellular hydrolysis may overestimate the actual rate of hydrolysis during the original incubation with the cell suspension.
While the release of intracellular peptidases may reflect the instability of *L. lactis* 1403 in the experimental system used, other studies in this laboratory have shown peptidase leakage to occur in milk-grown 1403 cells, which suggests that release of intracellular peptidases may be physiologically significant to the growth of this strain on milk caseins.

The third category contains strains 920, ML3, E8 and AM2. While these strains transport peptides intact, differences do exist in the relative rates of activity of their respective transport systems.

The di-/tripeptide system of *L. cremoris* AM2 had the lowest activity of the four strains tested and transported dipeptides and tripeptides at comparable rates. The di-/tripeptide transport system of *L. cremoris* E8 also showed similar rates for di- and tripeptides, although the rates of activity were twice those seen with strain AM2. In contrast the di-/tripeptide carrier system of *L. cremoris* ML3 transported dipeptides at much faster rates than tripeptides, while that of *L. lactis* 920 translocated tripeptides significantly faster than dipeptides.

Differences were also observed in the activities of the oligopeptide systems of strains E8, 920 and ML3. Whereas *L. cremoris* E8 appears to transport oligopeptides containing up to 8 residues as rapidly as di- or tripeptides, the transport of oligopeptides by *L. lactis* 920, while still comparable to di-/tripeptide transport rates, shows an inverse relationship with peptide size. The oligopeptide transport system of *L cremoris* ML3 did not transport the oligopeptide Val-Gly3 even though it was capable of translocating other peptide substrates such as tetra-alanine. This result suggests that significant differences may exist in the substrate specificities or affinities of the oligopeptide transport systems of different strains. Comparison of the relative rates of transport of dipeptides, tripeptides and oligopeptides by *L. cremoris* ML3 supports earlier proposals that the oligopeptide transport system of this strain is much less active than the di-/tripeptide carrier system (Smid, 1991).

The three categories proposed above are comparable to those suggested by Law (1977). Law failed to observe peptide uptake by *L. cremoris* SK11. He also found that, with respect to dipeptide uptake, lactococci differed as to whether they transported peptides intact, or after hydrolysis into their constitutive amino acids. However the strain

distribution of these two mechanisms of peptide utilisation does not reflect a fundamental distinction between *cremoris* and *lactis* strains, as proposed by Law (1977). In this study, both *L.lactis* and *L. cremoris* strains transported peptides intact. The extracellular hydrolysis of peptides by the *L. lactis* strain 1403 may simply reflect its tendency to autolyse. Lactococcal strains differ with respect to their autolytic properties (Crow *et al.*, 1993) and it may be coincidental that the *L. lactis* strains studied by Law were relatively autolytic. Furthermore, this study does not support the proposal of Law that the mechanism of dipeptide and tripeptide uptake in *L. cremoris* AM2 is significantly different to that of other *cremoris* strains.

The failure to observe competition for Val-Gly uptake in strain 920 is consistent with the results from a similar study carried out by Law (1977). From the finding that growth of *lactis* strains upon dipeptides as sole sources of amino acids was not inhibited by structurally related peptides, Law (1977) proposed that *L. lactis* strains preferentially hydrolyse dipeptides into amino acids for transport. The results of this study have shown, however, that dipeptide uptake in *L. lactis* 920 involves transport of the intact peptide. A possible, if somewhat improbable, explanation of the failure to observe competition between dipeptides for uptake in *L. lactis* 920, is that this strain has multiple di-/tripeptide carrier systems.

The results of this comparative strain study are therefore consistent with the current theory of lactococcal peptide transport which proposes the existence of a common transport system for both dipeptides and tripeptides, and an oligopeptide carrier system with affinity for peptide substrates greater than four residues. However, significant differences appear to exist between the activities and substrate specificities of the transport systems of starter strains. It is therefore not sound to make quantitative generalisations based on the study of one strain alone. This research has shown clearly that the ML3 strain is not typical of the lactococci as a group, and that the knowledge that has been obtained about the relative activities of the different peptide transport systems from the study of this strain does not necessarily apply to other *lactis* and *cremoris* strains.

One of the key objectives of dairy manufacturers is to engineer starters which accelerate ripening or produce modified flavours in cheeses. The existence of variations in the mechanisms by which different strains utilise milk caseins may complicate these attempts, as information obtained from the study of one strain employed in the manufacture of a particular cheese type, may not necessarily apply to a different strain used to produce another type of cheese.

6.5 Regulation of peptide metabolism

The success of bacteria in colonising environments lies largely in their ability to rapidly regulate their metabolic processes in response to changes in the levels of nutrients and wastes in those environments. This regulation can involve allosteric modification at the protein level, transcription regulation or translational regulation. Such mechanisms allow for the most appropriate allocation of cellular resources to the needs of a cell at any particular moment.

Limited information is available about what components of the lactococcal proteolytic system are nutritionally regulated. Exterkate (1985) demonstrated that low molecular weight peptides suppress the synthesis of the cell wall-associated proteinase. Presumably such regulation prevents unnecessary production of this enzyme when small oligopeptides are available as sources of essential amino acids.
The present study briefly addressed the question whether the other two important components of the proteolytic system, the internal peptidases and the peptide transport systems are also nutritionally regulated.

No significant differences in the activities of six intracellular peptidases were found when *L. lactis* 1403 was grown in media with different complements of amino acids and peptides. For example, there were no significant differences in dipeptidase or tripeptidase activities in culture grown on chemically defined media containing valine solely as the free amino acid, as a dipeptide, or as a tripeptide. These results imply that peptidase activity and/or expression in this strain is constitutive. This conclusion is in agreement with van Boven and Konings (1988) who found that dipeptidase activities were highly comparable between *L. cremoris* E8 cells grown on different media. This suggests that lactococcal peptidase activity in general is not nutritionally regulated.

Unlike the tripeptide permease system of *S. typhimurium* which is induceable, preliminary results from this study suggest that the di-/tripeptide transport system of *L. cremoris* E8 is not nutritionally regulated. No adaptive changes were observed in the *in vivo* activity of this transport system when cells were grown on a medium containing valine solely as a dipeptide, rather than in medium in which this essential amino acid was available as free valine. It is, of course, possible that this may be due to the fact that the Val-Gly dipeptide was supplied at a level above that which is growth limiting, so this conclusion must be regarded as tentative. Future work is needed to establish what nutrients if any regulate the expression and activity of the oligopeptide transport system and whether there are any significant strain differences in the processes of regulation.

6.6 Assessment of the importance of dipeptidase activity to peptide utilisation.

An important area of research in recent years has been the determination of which enzyme activities are essential for the utilisation of casein. Furthermore, there is much interest in establishing the complement of enzymes involved in cheese flavour development, especially those responsible for the bittering defect.
An unsuccessful attempt was made in this study to isolate a dipeptidase mutant of *L. cremoris* E8 to determine whether this intracellular enzyme was essential for the growth of this strain. Future work would be merited in this area, but the isolation of a dipeptidase mutant is probably better achieved using recently developed gene inactivation methods than the selection method employed in this research.

6.7 Future directions for research on peptide metabolism in the lactococci.

While the current study has helped answer some questions about lactococcal peptide metabolism, it has also generated some new questions which were beyond the reach of this thesis to address. Three areas in particular would merit further work.

The first of these areas would involve developing the whole-cell peptide uptake work using the β-casein oligopeptides, with the objective of determining an upper size limit for the oligopeptide transport system, and to obtain quantitative data for the translocation of these peptides. As these peptides represent more closely the natural substrates present in milk, they would also be useful for investigating whether the strain differences identified using a model peptide series are significant *in vivo*. In particular the utilisation of β-casein peptide 2 (QEPVLGPVRGPFPIIV) is of interest to industry as it has been identified as an important contributor to the bitterness defect that some strains produce in cheese (Shinoda *et al.*, 1985).

A further area meriting future work concerns developing the model proposed for the metabolism of casein-derived oligopeptides by *L. cremoris* E8. While this strain may be capable of utilising directly the large oligopeptides produced from the hydrolysis of the milk-caseins, the work of Smid *et al.* (1989b) has established that some essential amino acids must be transported solely as di- and tripeptides, as the di-/tripeptide carrier system was shown to be essential to the growth of lactococci on casein.
The failure to date to isolate endopeptidases or aminopeptidases with an extracellular location poses a question concerning the origin of these small peptides.

Further studies to investigate whether the cell wall-associated proteinase is capable of producing peptides considerably smaller than those identified to date are required. Alternatively, appropriate conditions for isolation of cell wall-located peptidases may yet be found.

Finally, more work is required to resolve the discrepancy between the failure to detect peptide utilisation in *L. cremoris* SK11 and the ability of this strain to grow well in milk.

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