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The proteolytic systems of lactic acid bacteria

Edmund R.S. Kunji[1], Igor Mierau[2], Anja Hagting[1], Bert Poolman[1] & Wil N. Konings[1]*
Departments of [1] Microbiology and [2] Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands; (author for correspondence)*

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Abstract

Proteolysis in dairy lactic acid bacteria has been studied in great detail by genetic, biochemical and ultrastructural methods. From these studies the picture emerges that the proteolytic systems of lactococci and lactobacilli are remarkably similar in their components and mode of action. The proteolytic system consists of an extracellularly located serine-proteinase, transport systems specific for di-tripeptides and oligopeptides (> 3 residues), and a multitude of intracellular peptidases. This review describes the properties and regulation of individual components as well as studies that have led to identification of their cellular localization. Targeted mutational techniques developed in recent years have made it possible to investigate the role of individual and combinations of enzymes *in vivo*. Based on these results as well as *in vitro* studies of the enzymes and transporters, a model for the proteolytic pathway is proposed. The main features are: (i) proteinases have a broad specificity and are capable of releasing a large number of different oligopeptides, of which a large fraction falls in the range of 4 to 8 amino acid residues; (ii) oligopeptide transport is the main route for nitrogen entry into the cell; (iii) all peptidases are located intracellularly and concerted action of peptidases is required for complete degradation of accumulated peptides.

Introduction

Lactic acid bacteria are used in the production of a wide range of dairy products such as cheeses and yoghurts. Several metabolic properties of lactic acid bacteria serve special functions which directly or indirectly have impact on processes such as flavour development and ripening of dairy products. The main functions are (i) fermentation and depletion of the milk sugar lactose; (ii) reduction of the redox potential; (iii) citrate fermentation and (iv) degradation of casein (Olsen, 1990). The degradation of caseins plays a crucial role in the development of texture and flavour. Certain peptides contribute to the formation of flavour, whereas others, undesirable bitter-tasting peptides, can lead to off-flavour. Detailed understanding of these processes may lead to engineered lactic acid bacteria with improved proteolytic properties.

It has been well-established that many lactic acid bacteria, isolated from milk products, are multiple amino acid auxotroph (Chopin, 1993). The requirement for amino acids is strain dependent and can vary from 4 up to 14 different amino acids. In milk, the amounts of free amino acids and peptides are very low. Lactic acid bacteria, therefore, depend for growth in milk on a proteolytic system that allows degradation of milk proteins (caseins) (Mills & Thomas, 1981; Juillard et al., 1995b). Caseins constitute about 80% of all proteins present in bovine milk. The four different types of caseins found in milk, α_{S1}-, α_{S2}-, β- and κ-casein, are organized in micelles to form soluble complexes (Schmidt, 1982). In free solution, caseins behave as non-compact and largely flexible molecules with a high proportion of residues accessible to the solvent, i.e., like random coil-type proteins (Holt & Sawyer, 1988). Caseins contain all amino acids necessary for growth of lactic acid bacteria in milk to high cell density, but it can be calculated that only a minor fraction of the total is actually needed (less than 1%).

The structural components of the proteolytic systems of lactic acid bacteria can be divided into three groups on the basis of their function: (i) proteinas-

es that breakdown caseins to peptides, (ii) peptidases that degrade peptides, and (iii) transport systems that translocate the breakdown products across the cytoplasmic membrane.

The proteinase is clearly involved in the initial degradation of caseins, yielding a large number of different oligopeptides. The initial analyses of the casein breakdown products liberated by the proteinases have indicated that, with a few exceptions, only large peptides are formed (Monnet et al., 1986; Visser et al., 1988; Monnet et al., 1989; Reid et al., 1991b; Pritchard & Coolbear, 1993). Consequently, further breakdown by extracellular peptidases was considered to be critical to fulfill the needs for essential and growth-stimulating amino acids. The external localization of proteinases is consistent with the finding that these are synthesized with a typical signal peptide sequence, but this property has not been found in any of the peptidases analysed so far (Kok & De Vos, 1994; Poolman et al., 1995). These findings are supported by biochemical and immunological data which indicate that the proteinases are present outside the cell, whereas most, if not all, peptidases are found in the cytoplasm.

These apparent discrepancies could be explained by: (i) the existence of extracellular peptidases which sofar have remained uncharacterized, possibly because most work has focussed on purification of soluble peptidases; (ii) a less restricted specificity of the cell envelope located proteinase; and/or (iii) the activity of membrane carriers capable of facilitating transport of peptides greater than 5–6 amino acid residues (Pritchard & Coolbear, 1993).

In this review, the list of putative components of the proteolytic pathway is updated and attempts are made to assign a physiological role to each of the enzymes. The main focus will be on the substrate specificity, expression/regulation and localization of the components. For detailed information on the biochemical properties of the enzymes, the cloning strategies and organization of the genes, the reader is referred to a series of reviews and the original references cited there (Tan et al., 1993a; Kok and De Vos, 1994; De Vos & Siezen, 1994; Poolman et al., 1995).

Most attention will be paid to the proteolytic system of lactococci, which is by far the best documented. The majority, if not all, of the enzymes necessary for degradation of caseins and transport of degradation products have been described. The second-best unravelled proteolytic systems are those of *Lactobacillus* (*Lb*) species, most notably *Lb. helveticus, Lb. delbrückii* and *Lb. casei*. Unfortunately, very little information is available on transport of casein breakdown products in these organisms. Whenever possible, similarities and differences between the proteolytic systems of lactococci and lactobacilli will be pointed out. Although work is advancing for other lactic actid bacteria, such as *Streptococcus, Pediococcus, Leuconostoc* and *Micrococcus* species, these data will not be discussed here.

In recent years, a series of elegant genetic tools have been developed for targeted inactivation of chromosomally located genes in lactococci and lactobacilli (Leenhouts 1991; Bhowmik et al., 1993; Leenhouts et al., 1996). These methods allowed, for the first time, the analysis of enzymes *in vivo* and have lead to a better understanding of the proteolytic pathway as a whole. Based on these and other results a model of the proteolytic pathway of *L. lactis* is presented, which accommodates most of the available data.

The proteinases of lactic acid bacteria

It has been well-established that degradation of caseins is initiated by a single cell wall-bound extracellular proteinase (PrtP) (Smid et al., 1991; Tan et al., 1993a; Pritchard & Coolbear, 1993; Kok & De Vos, 1994; De Vos & Siezen, 1994). The proteinases of many different lactic acid bacteria have been identified and characterized biochemically (Table 1). The biochemical and genetic properties, localization and specificity of the enzymes will be discussed in the following sections.

Genetic and biochemical properties of proteinases

The mature proteinase is a monomeric serine-proteinase with a molecular mass between 180–190 kDa, although breakdown products of smaller sizes are usually found upon isolation of the enzyme (Laan & Konings, 1989). The gene encoding PrtP has been cloned and sequenced for a number of *L. lactis* strains (Kok et al., 1988; Vos et al., 1989a; Kiwaki et al. 1989; Exterkate et al., 1993), *Lb. paracasei* (Holck & Næs, 1992) & *Lb. delbrückii* subsp. *bulgaricus* (Gilbert et al., 1996). The unprocessed proteinases of *Lb. paracasei* NCDO151, *L. lactis* Wg2 and NCDO763 consist of, 1902 amino acid residues, which compares well with the, 1946 residues of the *Lb. delbrückii* enzyme and the, 1962 residues for PrtP of *L. lactis* SK1, which have a duplication at the C-terminus (40 and 60 amino acids, respectively). The primary sequences of the lactococcal enzymes are more than 98% identical and more than 95% when compared to

Table 1. Proteinases of dairy lactic acid bacteria

Strain	Mw[a] (kDa)	Substrate[b]	Type[c]	pH[d]	Localization	Reference[g]
Lactococcus lactis subsp. *cremoris* WG2		κ-, β-casein	S		cell-wall[f]	Hugenholtz et al., 1987
	181				cell-wall[e]	Kok et al., 1988
Lactococcus lactis subsp. *cremoris* HP		κ-, β-casein	S	6.4	cell-wall[f]	Exterkate & De Veer, 1987a
Lactococcus lactis subsp. *cremoris* SK11	187	α_{s1}-, κ-, β-casein	S		cell-wall[e,f]	Vos et al., 1989a
Lactococcus lactis subsp. *cremoris* AC1		α_{s1}, κ-, β-casein			cell-wall[f]	Bockelmann et al., 1989
Lactococcus lactis subsp. *cremoris* AM1		α_{S1}-, κ-, β-casein			cell-wall[f]	Visser et al., 1991
Lactococcus lactis subsp. *cremoris* H2	180+	κ-, β-casein	S		cell-wall[f]	Coolbear et al., 1992
Lactococcus lactis subsp. *cremoris* NCDO763		α_{s1}-, κ-, β-casein	S	6.0	cell-wall[f]	Monnet et al., 1987
	181		S		cell-wall[e]	Kiwaki et al., 1989
Lactobacillus casei subsp. *casei* HN1		β-casein	S		cell-wall[f]	Kojic et al., 1991
Lactobacillus casei subsp. *casei* NCDO 151			S	6.5	cell-wall[f]	Næs & Nissen-Meyer, 1992
	181				cell-wall[e]	Holck & Næs, 1992
Lactobacillus delbrückii subsp. *bulgaricus* CNRZ 397	170+	α_{s1}-, β-casein	S[h]	5.5	cell-wall[f]	Laloi et al., 1991
			S		cell-wall[e]	Gilbert et al., 1996
Lactobacillus helveticus CNRZ 303		α_{s1}-, β-casein	S	7.5	cell-wall[f]	Zevaco & Gripon, 1988
Lactobacillus helveticus CP790	45+	α_{s1}-, β-casein	S	6.5	cell-wall[f]	Yamamoto et al., 1993
Lactobacillus helveticus L89	180+	α_{s1}-, β-casein	S	7.0	cell-wall[f]	Martín-Hernández et al., 1994

[a] If available, the molecular weight of the mature proteinase was calculated from the derived amino acid sequence of the cloned gene, otherwise as determined by gel filtration* or SDS-PAGE+. [b] Substrates degraded by the proteinase when tested. [c] Type of enzyme; S Serine-proteinase. [d] pH optimum of activity. [e] Localization as predicted from the presence of a signal or membrane anchor sequence. [f] Localization as predicted from fractionation studies and immuno-gold labeling. [g] Key references are only cited. [h] 45% of activity was recovered after incubation with 1 mM phenylmethylsulphonyl fluoride.

A
R E L E E L N V P G E I V E Z L Z Z E E S I T R I N K K I E K F Q Z E E Q Q Q T E D E L Q D K I H P F A Q T
Q S L V Y P F P G P I P N S L P Q N I P P L T Q T P V V V P P F L Q P E V M G V S K V K E A M A P K H K E M P
F P K Y P V Q P F T E S Q S L T L T D V E N L H L P L P L L Q S M M H Q P H Q P L P P T V M F P P Q S V L S L
S Q S K V L P V P Q K A V P Y P Q R D M P I I Q A F L L Y Q Q P V L G P V R G P F P I I V
L. lactis SK11, AM1 (a)
L. lactis NCDO763 (d)
L. lactis WG2 (e)
L. lactis HP, H2 (g)
Lb. helveticus CNRZ303
Lb. helveticus CP790

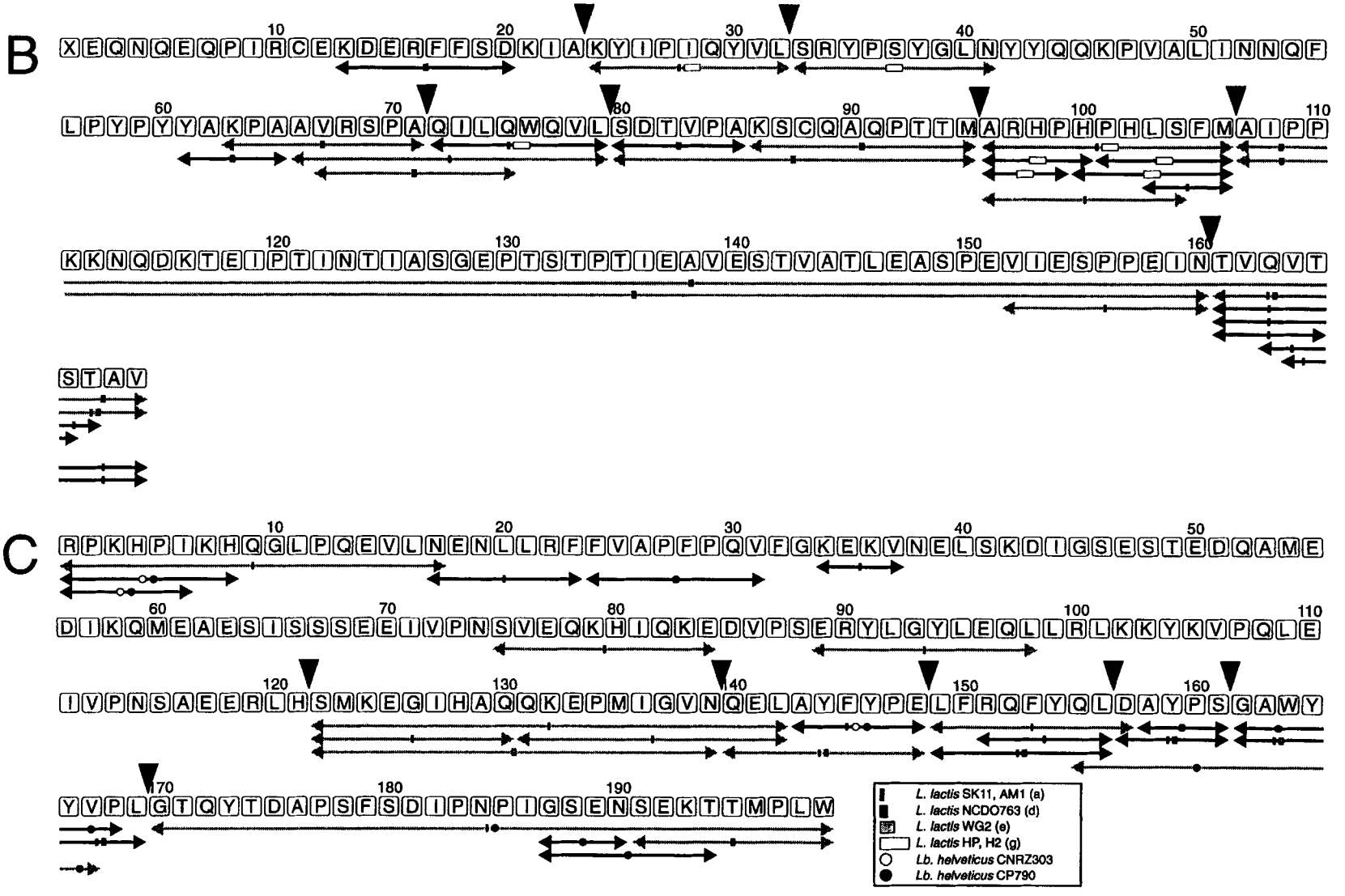

Figure 1. Primary sequences of β- (A), κ- (B) and α_{S1}-casein (C), and the peptides released by the activity of purified proteinases. Black arrows indicate degradation fragments of lengths of up to 8 amino acid residues (putative substrates of the oligopeptide transport system), while longer peptides are colored gray. The symbols relate to the strain or organism, in which the PrtP hydrolysate the particular peptide has been identified (shown in the legend of the figure). Black triangles show cleavage sites cut by all lactococcal proteinases and gray triangles indicate bonds hydrolyzed by the majority of the enzymes studied. (Sources: Monnet et al., 1986; Zevaco & Gripon, 1988; Visser et al., 1988; Monnet et al., 1989; Reid et al., 1991a; Reid et al., 1991b; Monnet et al., 1992; Yamamoto et al., 1993; Reid et al. 1994; Visser et al., 1994; Juillard et al., 1995a). Dashed arrows indicate peptides that are likely transported by the oligopeptide

the *Lb. paracasei* enzyme. The *Lb. delbrückii* enzyme shows up to 40% identity over the first 820 residues when compared to the lactococcal enzymes, while the C-terminal part does not share any homology. In *Lb. helveticus* CP790, a 45 kDa serine-proteinase has been identified, but sequence data are lacking to exclude the possibility that the isolated enzyme is an autoproteolytic product (Yamamoto et al., 1993). In another strain of this organism (L89), a proteinase of 180 kDa is found (Martín-Hernández et al., 1994).

Sequence alignments show that proteinases are related to subtilisins, which are serine-proteinases with similar catalytic domains (De Vos & Siezen, 1994). Comparison of the N-terminal sequence of the mature proteinase with that deduced from the nucleotide sequence revealed a typical signal sequence for Sec-dependent translocation and a prosequence, which both are removed by post-translational processing (Kok et al., 1988; Vos et al., 1989a; Kiwaki et al., 1989; Holck & Næs, 1992; Gilbert et al., 1996). The N-terminal part of the mature enzyme constitutes the catalytic domain (see below) and contains several conserved residues which are involved in catalysis and substrate positioning (Ser 433, His94, Asp30, Asn196 in the Sk11 enzyme) (De Vos & Siezen, 1994). The spacer that follows shows no homology to proteins of known function, but likely exposes the catalytic domain outside the cell-wall. The outermost C-terminal part is conserved in many surface proteins of Gram-positive bacteria and carries a sorting signal, typically LPXTG, followed by a putative membrane spanning α-helix and a small charged tail (Navarre & Schneewind, 1994). After translocation, the LPXT↓G sequence is cleaved (at the position indicated by the arrow) and the carboxyl group of threonine is probably covalently linked to a N-terminal glycine that is part of the cross-bridges in the peptidoglycan layer (Navarre & Schneewind, 1994).

In *L. lactis* Wg2, SK11 and *Lb. paracasei* NCDO 151 an enzyme has been identified which is involved in the maturation of the proteinase (PrtM) (Haandrikman et al., 1989; Vos et al., 1989b; Holck & Næs, 1992). The *prtM* gene product is also preceded by a signal sequence and has a consensus lipomodification site (Haandrikman et al., 1989; Vos et al. 1989b; Haandrikman et al., 1991; Holck & Næs, 1992; Sankaran & Wu, 1994). PrtM bears 30% amino acid sequence identity to PrsA of *Bacillus subtilis*, and these enzymes may function as extracellular chaperones (Kontinen et al., 1991). To date, a homolog of PrtM has not been identified in *Lb. delbrückii* subsp. *bulgaricus* (Gilbert et al., 1996).

Localization of the proteinases

The extracellular location of PrtP is supported by various kinds of data. Firstly, the proteinase can be liberated from the cell-wall with minimal lysis by treating cells with Ca^{2+}-free buffers (Mills & Thomas, 1981) or lysozyme (Laloi et al., 1991; Coolbear et al., 1992). The largest size of lactococcal PrtP, detected after release in Ca^{2+}-free buffer, is 165 kDa, which is believed to be the product of an intramolecular autoproteolytic event (Laan & Konings, 1989). Treatment with lysozyme yields a product of 180 kDa, which is close to the predicted size of the mature proteinase deduced from the primary sequence. Secondly, electron microscopy of immuno-gold labelled PrtP has confirmed a localization of the proteinase in the cell wall (Hugenholtz et al., 1987). Thirdly, without exception the genes encoding proteinases specify a typical N-terminal signal sequence, which targets the protein to the outside of the cell (see above) (Kok & De Vos, 1994; De Vos & Siezen 1994).

Specificity classes of proteinases

On the basis of degradation patterns of α_{S1}-, β- and κ-caseins, two proteinase specificity-classes have initially been described in lactococci, which are generally indicated as P_I and P_{III} (Visser et al., 1986). The primary substrates of P_I-type enzymes are β-casein and, to lesser extent, κ-casein, while P_{III}-type enzymes degrade α_{S1}-, β- and κ-caseins (Pritchard & Coolbear, 1993). Since P_I- and P_{III}-type proteinases from *Lactococcus* species are more than 98% identical, hybrid proteins could be constructed by swapping regions of the P_I-type proteinase of *L. lactis* subsp. *cremoris* Wg2 and the P_{III}-type enzyme of strain SK11. These studies limited the differences in the proteinase specificities to two regions (Vos et al., 1991). Substrate binding studies and computer modelling, based on the three-dimensional structure of subtilisins, suggest that the first region, with residues 131, 138, 142, 144 and 166 of SK11, is part of the substrate binding pocket of PrtP. The second region, in which particularly residues 747 and 748 are important, might be involved in electrostatic interactions with caseins.

The specificity of proteinases has been analysed further using the enzymes isolated from 16 different *L. lactis* strains and fragment 1-23 of α_{S1}-casein as substrate (Exterkate et al., 1993). On the basis of these

Table 2. Classification of proteinases according to the specificities toward α_{S1}-casein fragment 1–23

Group	Strains	Substr.	Cleavage sites in α_{s1}-casein fragment 1-23 [a]	Amino acid substitutions at positions relevant for substrate binding [b]								
			5 10 15 20 R P K H P I K H Q G L P Q E V L N E N L L R F	131	138	142	144	166	177	747	748	763
	L. lactis											
a	AM1, SK11, US3	α_{s1}, β, κ		Ser	Lys	Ala	Val	Asn	Leu	Arg	Lys	Asn
b	AM2			Thr	Thr	Ala	Leu	Asp	Leu	Arg	Lys	Asn
c	E8	α_{s1}, β, κ		Thr	Thr	Ala	Leu	Asp	Ile	Arg	Lys	Asn
d	NCDO763, UC317	α_{s1}, β, κ		Thr	Thr	Ala	Leu	Asp	Leu	Arg	Lys	His
e	WG2, C13, KH	β, κ		Thr	Thr	Ser	Leu	Asp	Leu	Leu	Thr	Asn
f	Z8, H61, TR, FD27	α_{s1}, β, κ		Thr	Thr	Ala	Leu	Asp	Leu	Leu	Thr	His
g	HP	β, κ		Thr	Thr	Asp	Leu	Asp	Ile	Leu	Thr	His
	Lb. paracasei											
	NCDO151			Thr	Thr	Ala	Leu	Asp	Leu	Gln	Thr	Asn
	Lb. bulgaricus											
	NCDO1489			Ser	Gly	Asp	Ile	Val		Gly	Thr	
	Lb. helveticus											
	L89											

[a] The main cleavage sites are indicated by arrows; the sizes of the arrows are related to the relative cleavage rates. [b] Numbering is according to the sequence of the SK11 proteinase (Vos et al., 1989a). Amino acid substitutions at positions relevant for substrate binding (Vos et al., 1991) are indicated. (Data according to Holck & Næs, 1992; Exterkate et al. 1993; Martín-Hernández et al., 1994; Gilbert et al., 1996.)

studies, the lactococcal proteinases were classified into seven groups, which displayed a whole range of different specificities rather than two extremes, i.e., a P_I- and P_{III}-type. Table 2 presents the identified specificity classes of lactococcal proteinases and the amino acid substitutions that are thought to be responsible for the observed phenotypes. It is evident that the subtle changes in specificity do occur as a result of minor genetic variations in the structural gene of PrtP. The catalytic domain of PrtP is not only highly conserved among lactococcal species, but also when compared to homologs in lactobacilli. The *Lb. paracasei* enzyme differs from the *L. lactis* Wg2 proteinase in only two positions, that are regarded as important for substrate specificity and binding (Holck & Næs, 1992) (Table 2). The *Lb. delbrückii* proteinase has distinct substitutions at positions 138 (Gly), 166 (Val) and 747 (Gly), and similar residues at others. Finally, the 180 kDa serine-proteinase of *Lb. helveticus* L89 essentially cleaves the same bonds as the lactococcal proteinases, but the relative amounts of fragments are clearly different (Martín-Hernández et al., 1994).

Casein degradation products

β-casein. The products resulting from the action of proteinases on β-casein have been analysed *in vitro* using purified enzymes of different *L. lactis* (Monnet et al. 1986; Visser et al., 1988; Monnet et al., 1989; Reid et al. 1991b) and *Lb. helveticus* strains (Zevaco & Gripon, 1988; Yamamoto et al., 1993) (Figure 1A). After separation of the proteolytic products by liquid chromatography, the different peptides were collected, purified further when necessary, and identified by Edman degradation and/or amino acid composition analysis. In some cases, additional information was obtained from mass-spectrometrical analysis of the purified peptide. These studies indicate that only part of β-casein is degraded and that relatively large fragments – only a few contain less than 8 amino acid residues – are formed. However, inspection of the HPLC-profiles shows that only the most abundant peptides have been analysed. Recently, more than 95% of the peptides formed by the action of the proteinase of *L. lactis* subsp. *cremoris* Wg2 on β-casein have been recovered using liquid chromatography in combination with on-line ion-spray mass spectrometry (Juillard et al., 1995a). The results show that β-casein is degraded by PrtP into more than hundred different oligopeptides ranging from 4 to 30 residues, of which a major fraction falls in the range of 4–10 residues (Figure 1A). The proteinase activity does not yield detectable amounts of di- and tripeptides, and only traces of phenylalanine were measured. More than 50% of the peptides originate from the C-terminal part of β-casein, while about half of the remaining peptides are derived from the 60–105 region.

The peptides which are liberated from β-casein by the proteinase of *L. lactis* SK11 and AM1 (P_{III}-type), NCDO763 and Wg2 (intermediate-types) and HP, H2 (P_I-type) are indicated in Figure 1A. In total, thirteen bonds are cleaved systematically by all lactococcal enzymes studied to date (indicated as black triangles in Figure 1A), and an additional six bonds are cleaved by most enzymes (indicated as gray triangles in Figure 1A). The majority of these bonds are located in the C-terminal part of β-casein. The peptides liberated by hydrolysis of these bonds constitute the major fraction in hydrolysates and are present during the earliest times of degradation (Juillard et al., 1995a; Fang & Kunji unpublished results). These peptides are likely to be the main suppliers of amino acids during growth on β-casein (see below). In addition to these fragments, all types of enzymes produce a large number of different small oligopeptides (black arrows in Figure 1).

All bonds cleaved by the action of the proteinase of *Lb. helveticus* CNRZ 303 on β-casein are also hydrolysed by the lactococcal proteinases (Zevaco & Gripon, 1988). Again, the major products are derived from the C-terminal region of β-casein. In *Lb. helveticus* CP790 a considerably smaller cell-wall bound proteinase has been identified, i.e. 45 versus 180 kDa (Yamamoto et al., 1993), which is claimed to be a complete serine proteinase and not the product of an autoproteolytic event. Nonetheless, the major degradation products of this enzyme are virtually identical to that of the CRNZ303 enzyme and the lactococcal proteinases with respect to peptides of small molecular weights (Figure 1A).

κ-casein. The product formation from κ-casein has been studied for the proteinases of *L. lactis* NCDO763, SK11, H2 and AM1, but only the major products have been identified sofar (Monnet et al., 1992; Reid et al. 1994; Visser et al., 1994). This milk protein is hydrolysed by each of the enzymes, albeit with different degradation patterns even after 24 hours of incubation (Reid et al. 1994). Many bonds are systematically hydrolysed by all types, but also several type-dependent cleavage sites were reported (Figure 1B). However, inspection of the HPLC profiles of the original papers suggests that the same peptides might

be present in each hydrolysate, although in different amounts. The degradation of κ-casein yields a large number of small oligopeptides, which originate mainly from region 96–106 and the C-terminal part.

α_{S1}-casein. Degradation of α_{S1} and α_{S2}-casein is confined to P_{III}-type and intermediate-type proteinases, while P_I-type proteinases cannot hydrolyse this substrate (Visser et al. 1986; Bockelmann et al., 1989). However, this conclusion is based on SDS-PAGE and not on the more sensitive HPLC analysis that has been used to characterize the degradation of β- and κ-casein. About 25 major oligopeptides were identified in the product formation of α_{S1}-casein by several proteinases, of which about half originate from the C-terminal region (Figure 1C) (Reid et al., 1991a; Monnet et al., 1992). Again, several different small oligopeptides are found in the hydrolysates of the various enzymes, of which several are bordered by preferential cleavage sites.

Cleavage sites. Various researchers have attempted to define specificity rules for the proteinase based on statistical analysis of cleavage site residues and putative interaction of these residues with the PrtP binding pocket (Monnet et al. 1992; Vos et al., 1991; De Vos & Siezen, 1994; Juillard et al., 1995a). It is apparent from inspection of Figure 1 that the proteinases have a very broad substrate specificity. On the basis of our recent studies, in which more than 95% of the β-casein degradation products have been identified, it is not possible to define unequivocally a consensus cleavage site. However, it is apparent that particular bonds are preferentially hydrolysed if the peptide product formation of different proteinases is compared. In addition to the specificity of PrtP *per se*, intrinsic properties of caseins might also play a role in degradation. Caseins have been described to behave, in free solution, as non-compact and largely flexible molecules with a high proportion of residues accessible to the solvent (Holt & Sawyer, 1988). Nonetheless, circular dichroism and Raman spectral studies have shown that β-casein contains about 12% α-helical structure (Swaisgood, 1993). Secondary structure predictions indicate that region 21–38 of β-casein has the potential to form α-helices, i.e. in the proximity of the phosphorylation sites. The same region has high sequence identity to the C-terminal part of hen egg-white riboflavin-binding protein (Holt & Sawyer, 1988). Possibly, the inability of the proteinases to degrade this part of the N-terminal region is related to the presence of α-helical structure.

It is striking that in several regions almost each peptide bond is cleaved by all types of proteinases, such as in regions 160–170 and, 190–195 in β-casein, while in other regions the bonds are only incidently cut. This could reflect the broad specificity of PrtP, but can also be attributed to a better accessibility of particular loops for degradation. Furthermore, the hydrolysis of casein by PrtP is a complex process, in which parameters such as refolding of the substrate after cleavage and aggregation of casein molecules may also play a role.

In conclusion, the biochemical properties of the proteinases of the various lactic acid bacteria are very similar, i.e. most enzymes, if not all, are serine-proteinases of similar size (Table 1). Sequence comparisons reveal a remarkably high degree of identity, even when proteinases of different specificity classes or organisms are compared. Also the product formation seems to cross borders of specificity classes and species, which is apparent from the large number of preferential cleavage sites. Many different small oligopeptides (4–8 amino acid residues) are formed, which contain all essential and growth-stimulating amino acids, and many of these peptides are produced in high amounts. To date no significant amounts of free amino acids and di/tripeptides have been detected in hydrolysates formed by the various proteinases.

Amino acid and peptide transport systems

To utilize amino acids for biosynthesis, degradation products derived from casein have to traverse the membrane at one stage or another. In the following sections, the transport processes are reviewed with the emphasis on peptide transport systems.

Lactococcal amino acid transport systems

Lactococci possess at least 10 amino acid transport systems which have a high specificity for structurally similar amino acids, e.g. Glu/Gln, Leu/Ile/Val, Ser/Thr, Ala/Gly, Lys/Arg/Orn (Konings et al., 1989). Several amino acid transport systems were characterized as being driven by hydrolysis of ATP, i.e. those for Glu/Gln, Asn and Pro/Glycine-Betaine (Konings et al., 1989; Poolman 1993; Molenaar et al., 1993). The amino acid transport systems for Leu/Val/Ile, Ala/Gly, Ser/Thr and Met are driven by the proton motive force, whereas the Arg/Orn antiporter is driven by the concentration gradient of both solutes (Konings et al., 1989). To date, genes encoding lactococcal amino acid transport systems have not been cloned.

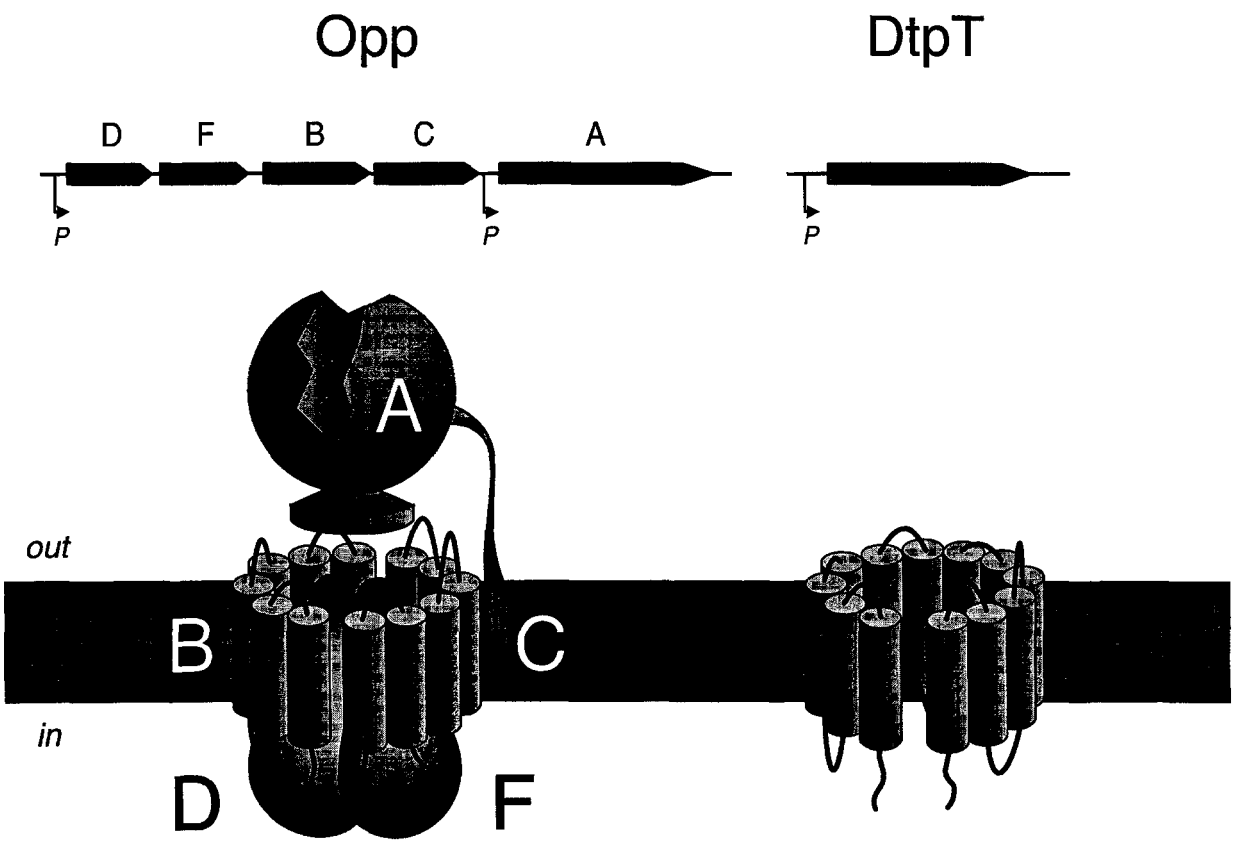

Figure 2. Model and gene organization of the oligopeptide transport system (Opp) and di/tripeptide transporter (DtpT) of *Lactococcus lactis*. (Data from Tynkkynen et al., 1993; Hagting et al., 1994.)

The lactococcal di/tripeptide transporter for hydrophilic substrates (DtpT)

Transport studies in peptidase-free membrane vesicles of *L. lactis* have shown that relatively hydrophilic di- and tripeptides are transported by a proton motive force-driven transport mechanism (Smid et al., 1989). The gene (*dtpT*) encoding this di-tripeptide transport protein has been cloned by complementation and functionally expressed in *E. coli* and *L. lactis* (Hagting et al., 1994). The amino acid sequence deduced from the nucleotide sequence of *dtpT* shows no significant similarity to other known bacterial peptide transport systems, which all belong to the ABC superfamily (see below) and couple transport to the hydrolysis of ATP (Higgins, 1992). In fact, DtpT belongs to a new family of proton motive force-driven peptide transport systems, called the PRT-family, which sofar has only eukaryotic counterparts of DtpT (Steiner et al., 1995). Recent database searches have revealed bacterial open reading frames (ORFs) with significant similarity to DtpT (unpublished results). One of these ORFs correspond to a partial sequence located downstream of aminopeptidase N gene of *Lb. helveticus* (Christensen et al., 1995). The secondary structure of DtpT is similar to that of proton motive force driven secondary transport proteins and consists of twelve putative membrane-spanning α-helices (Figure 2). Using flanking regions of the gene, *dtpT* has been deleted from the chromosome via homologous recombination. Characterisation of the Δ*dtpT* strain indicates that DtpT is the only transport protein for hydrophilic di- and tripeptides in *L. lactis*. Its role in the uptake of casein degradation products is discussed below (section: The role of peptide transport systems *in vivo*).

The lactococcal oligopeptide transport system (Opp)

From mutant analysis it has become apparent that *L. lactis* also possesses a transporter that is specific for oligopeptides (Opp) (Kunji et al., 1993). Growth experiments in chemically defined media containing

peptides of varying length have suggested that Opp transports peptides up to lengths of 8 residues (Tynkkynen et al., 1993). Preliminary experiments, in which translocation of peptides formed by the action of PrtP on β-casein was analysed, indicate that oligopeptides consisting of up to 10 amino acids may be transported (Fang & Kunji, unpublished results).

On the basis of metabolic inhibitor studies it has been concluded that oligopeptide transport is driven by ATP rather than the proton motive force (Kunji et al., 1993). The genes encoding the oligopeptide transport protein have been cloned, sequenced and functionally expressed in strains of *L. lactis* (Tynkkynen et al., 1993). Five open reading frames correspond to polypeptides that are typical components of binding protein-dependent transport systems (OppDFBCA) (Figure 2), and on the basis of sequence comparisons the system has been classified as a member of the Binding Cassette (ABC) superfamily (Higgins, 1992). The sixth gene of the operon, that is located 5′ of *oppA*, encodes the endopeptidase PepO (Mierau et al., 1993; Tynkkynen et al., 1993). The five subunits of the oligopeptide transport system include a peptide binding protein (OppA), two integral membrane proteins (OppB and OppC), and two ATP-binding proteins (OppD and OppF) (Figure 2). The derived amino acid sequence of the *oppA* gene has a consensus prolipoprotein cleavage site Leu-Ser-Ala↓Cys (Tynkkynen et al., 1993), which, through fatty acid modification of the N-terminal cysteine, anchors the mature protein at the outer surface of the cytoplasmic membrane (Von Heijne, 1989; Sankaran and Wu, 1994; Detmers & Kunji unpublished results).

OppA serves as the receptor protein that delivers peptides to the membrane-bound translocator complex. Elucidation of the tertiary structure of OppA of *Salmonella typhimurium* indicated that the protein is composed of three domains: Domains I and III are involved in the binding of oligopeptides in the manner of a Venus fly-trap; and domain II is typical for peptide binding proteins but its function has not been established (Figure 3) (Tame et al., 1994). The structure has shed light on the intriguing observation that OppA is able to bind peptides with high affinity, which differ in size and amino acid composition (Tame et al., 1994). It turns out that the peptide backbone is bound to the binding pocket of OppA through salt bridges and hydrogen bonds, while the side chains of the peptide are accommodated in large pockets with minimal interaction with OppA. The N-terminal end of the peptide is bound to Asp419, while the C-terminal end is bound to Arg413, His371 or Lys307 in case of a tri-, tetra- or pentapeptide, respectively (Figure 3). The same principal has been observed in the binding of dipeptides to the dipeptide binding protein of *E. coli* (Dunten & Mowbray, 1995). OppA of *S. typhimurium* and *L. lactis* are homologous, but the identity between the two genes is only 25%. The lactococcal OppA is about 60 amino acids larger and might have an extra loop at the N-terminus which could function as a spacer to connect the protein to its membrane anchor (Figure 3).

OppB and OppC are highly hydrophobic proteins that, on the basis of hydropathy profiling, are able to span the cytoplasmic membrane in α-helical configuration six times (Figure 2). These proteins are likely to constitute the pathway that facilitates the translocation of oligopeptides across the membrane. OppD and OppF are homologous to the ATP binding protein(s) (domains) of the ABC-transporter superfamily (Higgins, 1992). These proteins most likely couple the hydrolysis of ATP to conformational changes in OppB/C that allow passage of the peptides across the membrane (Figure 2).

To discriminate between OppDFBCA and PepO as essential components of the proteolytic pathway of *L. lactis*, two integration mutants have been constructed, one defective in OppA and the other in PepO. Growth of these mutants in milk and in a chemically defined medium with oligopeptides has shown that the OppDFBCA system, but not the endopeptidase, is essential for the utilization of milk proteins and oligopeptides (Tynkkynen et al., 1993).

In *E. coli* and *S. typhimurium* peptides of 3 to 6 amino acids are transported by Opp. Translocation of longer peptides in Gram-negative bacteria may be restricted by the upper size exclusion limits of the outer membrane pores rather than the transporter (Payne & Smith, 1994). Moreover, the upper size limits of Opp have not been studied systematically. Since the number of amino acid combinations increases rapidly with the size of the peptide, it is difficult to explore the upper size exclusion range without taking into account the specificity. One approach to solve this problem is to use random libraries of peptides, which contain all amino acids in random order but do have a specified size (Momburg et al., 1994). The other approach we are currently taking is the investigation of transport of natural substrates, i.e. fragments of various lengths liberated from β-casein by the action of the proteinase. This is done by following the uptake of casein degradation products *in vivo* by ion-spray mass spectrometry

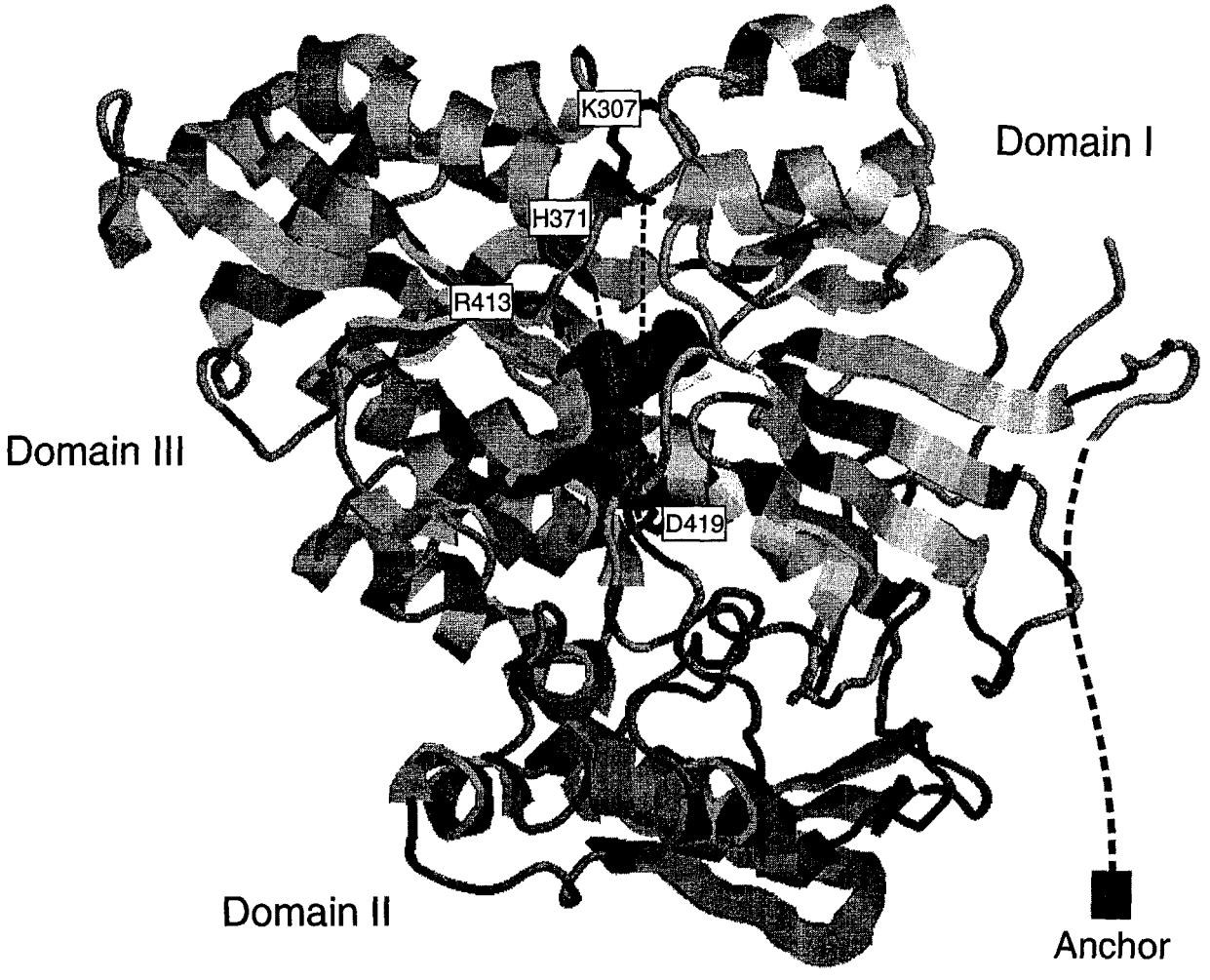

Figure 3. Tertiary structure of the oligopeptide binding protein (OppA) of *Salmonella typhimurium*. The identical residues of OppA of *Lactococcus lactis* are colored black and Van der Waals spheres depict the bound substrate tri-lysine. The amino acid residues, essential for binding of the peptide, are indicated in boxes and the direction of the substrate backbone is depicted by a dotted line. Also indicated is the position of the putative spacer which connects the membrane anchor to OppA of *L. lactis*. (Data from Tynkkynen et al., 1993; Tame et al., 1994.)

and by analysing the transport of chemically synthesized peptides *in vitro*.

The lactococcal di/tripeptide transport system for hydrophobic substrates (DtpP)

Mutants defective in Opp and DtpT are impaired in their ability to utilize hydrophilic di/tripeptides and oligopeptides, but are still capable of using a wide range of hydrophobic di- and tripeptides. This observation led to the discovery of a third peptide transport system, designated DtpP (Foucaud et al., 1995). Inhibitor studies have indicated that this system is driven by hydrolysis of ATP, which is the same mechanism of energy coupling as described for Opp. The genes encoding DtpP have not been cloned, but inhibition by *ortho*-vanadate suggests that it also belongs to the family of ATP-dependent transporters (Higgins, 1992).

Amino acid and peptide transport systems in lactobacilli

While a lot is known about amino acid and peptide transport in lactococci, virtually no information is available on similar systems in lactobacilli. Preliminary results suggest that the amino acid transport systems of *Lb. helveticus* are similar to those of *L. lac-*

tis, because the mechanism of uptake is identical for the same amino acids (Nakajima, unpublished results). The gene coding for a branched chain amino acid carrier (*brnQ*) of *Lb. delbrückii* subsp. *lactis* has been cloned and sequenced (Stucky et al., 1995a). Like its lactococcal counterpart, this branched chain amino acid carrier is also driven by the proton motive force. Recently, the genes encoding a transporter specific for aromatic amino acids (*aroP*) and one for dipeptides (*dppE*) have been cloned and sequenced in this organism (Vongerichten and Krüger, unpublished results).

As indicated above, a homolog of DtpT is specified by a sequence located downstream of *pepN* in *Lb. helveticus* (Christensen et al., 1995). Transport experiments have shown that substrates, typical for the lactococcal DtpT, are indeed transported by this organism. Preliminary experiments also indicate that an oligopeptide transport system is present in *Lb. helveticus* (Nakajima, unpublished results).

Peptidases of lactic acid bacteria

Following breakdown by PrtP and/or uptake, the casein-derived peptides need to be hydrolysed further by peptidases. The specificity of individual peptidases, their cellular location and their role in the utilization of caseins and peptides will be discussed in the following sections. The discussion of the peptidase specificities is confined to those enzymes which have been purified to homogeneity and characterized biochemically. The genes specifying most of these enzymes are available and biochemical data, sequence alignments, substrate specificities and immunological data are used to compare enzymes of different organisms.

A true comparison of the specificities of peptidases is only possible when well-designed sets of peptides are used. Unfortunately, most studies are inspired by the availability of peptides in the laboratory freezers and a systematic approach has been chosen in only a few cases. Nonetheless, some generalizations can be made, but the set of data is insufficient for true statistical evaluation. Many authors have claimed differences in specificity, but these could well be minor variations on a general theme. Tables 3 and 4 summarize general biochemical and genetic properties of the best characterized peptidases of lactococci and lactobacilli.

General aminopeptidases

Aminopeptidase N. For aminopeptidase N (PepN), there is a clear consensus about the biochemical properties of the enzyme. In most, if not all, organisms studied, the enzyme is a monomeric metallopeptidase of about 95 kDa. Sequence alignments have shown that the gene is conserved among dairy lactic acid bacteria; the primary sequence of PepN of *Lb. helveticus* is 72% and 49% identical to the enzyme of *Lb. delbrückii* subsp. *lactis* and *L. lactis* subsp. *cremoris*, respectively (Tan et al. 1992a; Strøman, 1992; Klein et al., 1993; Christensen et al., 1995). In addition, the primary sequence of *pepN* is homologous to the mammalian aminopeptidase N. The conserved signature sequence F–GAMEN-G indicates that PepN belongs to the subclass of zinc-dependent metallo-peptidases.

PepN is capable of cleaving N-terminal amino acids from a wide range of peptides differing both in size and composition. In most papers, the specificity of the enzyme for di- and tripeptides has been investigated, but as will be shown below, PepN activity is also directed towards oligopeptides (Tan et al., 1990; Miyakawa et al. 1992; Baankreis, 1992; Arora & Lee, 1992; Tan et al. 1993b; Niven et al., 1995; Sasaki et al., 1996a). Generally, dipeptides containing Pro in either two positions are not cleaved, while tripeptides which contain Pro in either first or second position are hydrolysed (Tan et al., 1990; Miyakawa et al., 1992; Arora & Lee, 1992; Tan et al. 1993b). The enzyme of *L. lactis* shows a marked preference for dipeptides containing Arg as the N-terminal residue, but, to a lesser extent, is also capable of cleaving other residues such as Lys & Leu (Niven et al., 1995). An increase in activity is observed with increasing hydrophobicity of the C-terminal residue of the dipeptide Arg-X (Niven et al., 1995). A similar relationship is observed for the hydrolysis of Ala-X and Leu-X peptides by PepN of *Lb. helveticus* (Miyakawa et al., 1992). Tripeptides are also readily cleaved by PepN, but information about preferred peptides is not available.

Three different studies have reported the hydrolysis of oligopeptides by PepN of *L. lactis* (Baankreis, 1992; Tan et al., 1993b; Niven et al., 1995). First, in a study in which a tryptic digest of β-casein was incubated with purified PepN, oligopeptides ranging from 4 to 12 acid residues were hydrolysed (Tan et al., 1993b); several of these fragments contained Pro and even Pro-X-Pro sequences. Second, a *PepN* mutant was found to be impaired in its ability to hydrolyse peptides like $(Lys)_4$, $(Lys)_5$ and $(Lys)_3$ $Trp(Lys)_3$, while di- and tripeptide hydrolysis was unaffected due to activity of other peptidases (Baankreis, 1992). Apparently, these oligopeptides are not hydrolysed by endopeptidases present in this organism. Third, several peptides with

Table 3. General aminopeptidases of dairy lactic acid bacteria

	Name	Substrate n = 1, 2, 3 . . .	Strain	Mw[a] kDa	Quat. struct.	Type[b] e[b]	pH Optimum [c]	Localization	Reference
Aminopeptidase N	PepN	X↓(X)$_n$	*Lactococcus lactis* subsp. *cremoris* Wg2	95*	mono	M	7		Tan & Konings, 1990
			Lactococcus lactis subsp. *cremoris* MG1363	95*				intracellular[d,e]	Van Alen-Boerighter et al., 1991
			Lactococcus lactis subsp. *cremoris* HP	95*				intracellular[e]	Exterkate et al., 1992
			Lactococcus lactis subsp. *cremoris* Wg2	95				intracellular[d]	Strøman, 1992
			Lactococcus lactis subsp. *cremoris* Wg2	95				intracellular[d]	Tan et al., 1992a
			Lactococcus lactis subsp. *cremoris* Wg2					intracellular[e]	Tan et al., 1992b
			Lactobacillus casei subsp. *casei* LGG	87+	mono	M	7		Arora & Lee, 1992
			Lactobacillus delbrückii subsp. *lactis* DSM7290	95	mono	M	7	intracellular[d]	Klein et al., 1993
			Lactobacillus delbrückii subsp. *bulgaricus* B14	95*	mono	M	7		Bockelmann et al., 1992
			Lactobacillus helveticus LHE511	92*	mono	M	7	cell-wall[e]	Miyakawa et al., 1992
			Lactobacillus helveticus CNRZ32	97*	mono		6.5		Khalid & Marth, 1990a
			Lactobacillus helveticus ITGL1	97*	mono	M	6.5	cell-wall[e]	Blanc et al., 1993
			Lactobacillus helveticus CNRZ32	97				intracellular[d]	Varmanen et al., 1994
			Lactobacillus helveticus CNRZ32	97				intracellular[d]	Christensen et al., 1995
			Lactobacillus helveticus SBT2171	95*	mono	M	7		Sasaki et al., 1996a
			Lactobacillus helveticus SBT2171					intracellular[e]	Bosman et al., 1996
Aminopeptidase C	PepC	X↓(X)$_n$	*Lactococcus lactis* subsp. *cremoris* AM2	50*	hexa	T	7	intracellular[e]	Neviani et al., 1989
			Lactococcus lactis subsp. *cremoris* Wg2					intracellular[e]	Tan et al., 1992b
			Lactococcus lactis subsp. *cremoris* AM2	50				intracellular[d]	Chapot-Chartier et al., 1993
			Lactobacillus delbrückii subsp. *lactis* DSM7290	51		T		intracellular[d]	Klein et al., 1994a
			Lactobacillus delbrückii subsp. *bulgaricus* B14	54*	tetra	T	7		Wohlrab & Bockelmann, 1993
			Lactobacillus helveticus CNRZ32	50		T		intracellular[d]	Fernández et al., 1994
			Lactobacillus helveticus CNRZ32	51		T		intracellular[d]	Vesanto et al., 1994
Tripeptidase	PepT	X↓X–X	*Lactococcus lactis* subsp. *cremoris* Wg2	52*	di	M	7.5		Bosman et al., 1990
			Lactococcus lactis subsp. *cremoris* Wg2					intracellular[e]	Tan et al., 1992b
			Lactococcus lactis subsp. *cremoris* AM2	52*	di	M	7.5	intracellular[e]	Bacon et al., 1993
			Lactococcus lactis subsp. *cremoris* MG1363	46				intracellular[d]	Mierau et al., 1994
	53	X↓X–X	*Lactococcus lactis* subsp. *cremoris* IMN-C12	23+	tri	T	5.8	cell-wall[e]	Sahlstrøm et al., 1993

Table 3. Continued

	Name	Substrate n = 1, 2, 3 . . .	Strain	Mw[a] kDa	Quat. struct.	Type[b] e[b]	pH Optimum [c]	Localization	Reference
Dipeptidase	PepV	X↓X	*Lactococcus lactis* subsp. *cremoris* H61	50*	di	M	8	intracellular[e]	Hwang et al., 1981
			Lactococcus lactis subsp. *cremoris* Wg2	49*	mono		8	intracellular[e]	Van Bven et al., 1988
			Lactococcus lactis subsp. *cremoris* MG1363	51				intracellular[d]	Fayard and Mierau, 1996
			Lactobacillus delbrückii subsp. *lactis* DSM7290	52	–	M		intracellular[d]	Vongerichten et al., 1994
			Lactobacillus delbrückii subsp. *bulgaricus* B14	51	mono	M	7.5		Wohlrab & Bockelmann, 1992
			Lactobacillus helveticus SBT2171	50*	mono	M	8		Tan et al., 1995
			Lactobacillus helveticus SBT2171					intracellular[e]	Bosman et al., 1996
			Lactobacillus helveticus CNRZ32	50	mono	M		intracellular[d]	Shao et al., 1996
	PepD	X↓X	*Lactobacillus helveticus* CNRZ32	54				intracellular[d]	Dudley et al., 1996
			Lactobacillus helveticus 53/7	54	octo	T	6	intracellular[d]	Vesanto et al., 1996

[a] If available, the molecular weight of the monomer was calculated from the derived amino acid sequence of the cloned gene, otherwise as determined by gel filtration* or SDS-PAGE^{+}. [b] Type of enzyme; M Metallo-peptidase; S Serine-peptidase; T Thiol-peptidase. [c] pH optimum of activity. [d] Localization as predicted from the absence of a signal or membrane anchor sequence. [e] Localization as predicted from fractionation studies and immuno-gold labeling.

the formula Lys-Phe-$(Gly)_n$ were tested for hydrolysis by purified PepN. Using V_{max}/K_m as measure of the overall efficiency of degradation, it was shown that a hexamer is the optimal substrate for PepN (Niven et al., 1995). A recently characterized PepN of *Lb. helveticus* was also able to hydrolyse peptides of lengths up to 10 amino acid residues, even those containing Pro in first position (Sasaki et al., 1996a).

Aminopeptidase C. The general aminopeptidase C (PepC) is a thiol-peptidase of about 50 kDa in all organisms studied to date (Neviani et al., 1989; Chapot-Chartier et al., 1993; Wohlrab & Bockelmann, 1993; Klein et al., 1994a; Fernández et al., 1994; Vesanto et al., 1994). However, differences with respect to the quaternary structure have been reported, i.e. tetramer vs hexamer (Table 3). PepC is also highly conserved among dairy lactic acid bacteria. The amino acid identity of *Lb. helveticus* PepC is 48% and 73% compared to the enzymes of *L. lactis* subsp. *cremoris* and *Lb. delbrückii* subsp. *lactis*, respectively (Chapot-Chartier et al., 1993; Fernández et al., 1994; Klein et al., 1994a; Vesanto et al., 1994). PepC shows some similarity to mammalian bleomycin hydrolase, which is putatively involved in the degradation of the glycopeptide antibiotic bleomycin. PepC has recently been crystallized for structural determinations (Mistou et al., 1994).

Characterization of the substrate specificity of PepC has been largely confined to *para*-nitro-anilide or β-naphthylamide derivatives. In the case of *Lb. delbrückii* subsp. *lactis*, PepC has similar activities as PepN for substrates like Leu-β-NA and His-β-NA, but differs from PepN in its ability to hydrolyse Gly-β-NA, Asp-β-NA, Gly-Ala-β-NA and Gly-Phe-β-N (Klein et al., 1994a). This indicates that PepN and PepC have distinct as well as overlapping specificities. PepC of *Lb. delbrückii* subsp. *bulgaricus* hydrolyses a broad range of di- and tripeptides (Wohlrab & Bockelmann, 1993). Similar to PepN, dipeptides containing Pro are not cleaved, but Pro-containing tripeptides are to some extent. Some preference for dipeptides with Ala, Leu or Lys in N-terminal position has been observed. In the same study, a hexa- and pentapeptide are not hydrolysed by PepC, but the upper size limits of this enzyme have not been explored systematically.

Tripeptidases. Two peptidases capable of cleaving tripeptides have been identified in strains of *Lactococcus lactis* subsp. *cremoris*, but not in other dairy lactic acid bacteria. One tripeptidase (PepT) has been characterized in two different studies and the gene has been cloned and sequenced (Bosman et al., 1990; Bacon et al., 1993; Mierau et al., 1994). PepT is a metalloenzyme and a homodimer of about 105 kDa. Primary sequence alignments revealed that PepT is 47% identical to PepT of *S. typhimurium*. The other tripeptidase, designated peptidase 53, is a trimer of 72 kDa which has been claimed to be a thiol-enzyme (Sahlstrøm et al., 1993). Distinct pH optima for Leu-Leu-Leu hydrolysis by the two enzymes have been found, i.e. 7.5 and 5.8 for PepT and peptidase 53, respectively.

The specificity of PepT is strictly limited to tripeptides as no detectable activity for di- and oligopeptides is observed (Bosman et al., 1990; Bacon et al., 1993). PepT was characterized as having a broad specificity; even substrates such as tri-ornithine were degraded. Although not systematically investigated, PepT was found to hydrolyse tripeptides with Pro in first and third position, but is unable to cleave peptides having Pro in second position. With respect to substrate specificity, peptidase 53 is clearly distinct from PepT (Sahlstrøm et al., 1993). Peptidase 53 has a preference towards tripeptides, but is also able to cleave di- and oligopeptides, albeit with lower activity. Hydrolysis of Pro-containing peptides by peptidase 53 has not been investigated.

Dipeptidases. The general dipeptidase (PepV) is a monomeric metallopeptidase of about 51 kDa, although the enzyme isolated from *L. lactis* subsp. *cremoris* H61 has been reported to have a homodimeric quaternary structure (Hwang et al., 1981). The primary sequence of the dipeptidase of *L. lactis* subsp. *cremoris* and *Lb. delbrückii* subsp. *lactis* do not resemble the general dipeptidase PepD of *E. coli* (Vongerichten et al., 1994; Fayard & Mierau, 1996). Recently, the gene coding for a totally distinct dipeptidase, designated PepD or PepDA, has been cloned in two strains of *Lb. helveticus* (Dudley et al., 1996; Vesanto et al., 1996). Although similar in subunit size and specificity compared to PepV, PepD/PepDA has a distinct pH optimum (6 vs 8), quaternary structure (octomer vs monomer) and catalytic properties (thiol-peptidase vs metallopeptidase). In addition to PepD, *Lb. helveticus* strains also have a PepV-like enzyme, which has been purified and characterized (Tan et al., 1995). The deduced primary sequence of the recently cloned and sequenced *pepV* gene of *Lb. helveticus* CNRZ32 has 69% identity with the one from *Lb. delbrückii* (Shao et al., 1996).

The specificity of dipeptidases is really confined to dipeptides, since none of the tested tri- and oligopep-

Table 4. Proline-specific peptidases of dairy lactic acid bacteria

Enzyme	Name	Substrate n = 1, 2, 3 . . .	Strain	Mw[a] kDa	Quat. struct.	Type [b]	pH Opt [c]	Localization	Reference
Prolidase	PepQ	X↓Pro	*Lactococcus lactis* subsp. *cremoris*H61	43*	mono	M	7		Kaminogawa et al., 1984
			Lactococcus lactis subsp. *cremoris* AM2	42*	mono	M	8	intracellular[e]	Booth et al., 1990b
			Lactobacillus delbrückii subsp. *lactis* DSM7290	41	–	M	–	intracellular[d]	Stucky et al., 1995b
Aminopeptidase P	PepP	X↓Pro-$(X)_n$	*Lactococcus lactis* subsp. *cremoris* NCDO763	43*	mono	M	8	intracellular[e]	Mars & Monnet, 1995
X-prolyl-dipeptidyl aminopeptidase	PepX	X-Pro↓$(X)_n$	*Lactococcus lactis* subsp. *lactis* H1	83+	di	S		intracellular[d]	Lloyd and Pritchard, 1991
			Lactococcus lactis subsp. *cremoris* P8-2-47	90*	mono	S	7	cell-wall[e]	Kiefer-Partsch et al., 1989
			Lactococcus lactis subsp. *cremoris* NCDO763	88*	di	S	8.5	intracellular[e]	Zevaco et al., 1990
			Lactococcus lactis subsp. *cremoris* AM2	59*	di	S	7.5	intracellular[e]	Booth et al., 1990a
			Lactococcus lactis subsp. *cremoris* P8-2-47	88		S		intracellular[d]	Mayo et al., 1991
			Lactococcus lactis subsp. *cremoris* NCDO763	88				intracellular[d]	Nardi et al., 1991
			Lactococcus lactis subsp. *cremoris* nTR	88*	di	S	7.5	intracellular[e]	Yan et al., 1992
			Lactococcus lactis subsp. *cremoris* Wg2					intracellular[e]	Tan et al., 1992b
			Lactobacillus casei subsp. *casei* LLG	79+	mono	S	8		Habibi-Najafi & Lee, 1994
			Lactobacillus delbrückii subsp. *lactis* DSM7290	88	mono	S	7	intracellular[d]	Meyer-Barton et al., 1993
			Lactobacillus delbrückii subsp. *bulgaricus* CNRZ397	82*	di	S	7	intracellular[e]	Atlan et al., 1990
			Lactobacillus delbrückii subsp. *bulgaricus* B14	95+	di	S	6.5		Bockelmann et al., 1991
			Lactobacillus helveticus CNRZ32	72+	mono	S	7		Khalid & Marth, 1990b
			Lactobacillus helveticus 53/7	91	di	S	6.5	intracellular[d]	Vesanto et al., 1995a
			Lactobacillus helveticus CNRZ32	88		S		intracellular[d]	Yüksel & Steele, 1995
			Lactobacillus helveticus SBT2171	95*	di	(S)[f]	6.5		Sasaki et al., 1996b
			Lactobacillus helveticus SBT2171					intracellular[e]	Bosman et al., 1996
Prolinase	PepR	Pro↓X	*Lactobacillus helveticus* CNRZ32	35				intracellular[d]	Dudley & Steele, 1994
			Lactobacillus helveticus 53/7	35			7.5		Varmanen et al., 1996b
Proline iminopeptidase	PepI	Pro↓X–$(X)_n$	*Lactococcus lactis* subsp. *cremoris* HP	50+		M	8.5	intracellular[e]	Baankreis & Exterkate, 1991
			Lactobacillus delbrückii subsp. *lactis* DSM7290	33		S		intracellular[d]	Klein et al., 1994b
			Lactobacillus delbrückii subsp. *bulgaricus* CNRZ397	33		S		intracellular[d]	Atlan et al., 1994
			Lactobacillus delbrückii subsp. *bulgaricus* CNRZ397		tri		6.5	cell-wall[e]	Gilbert et al., 1994
			Lactobacillus helveticus 53/7	34	di	S		intracellular[d]	Varmanen et al., 1996a

[a] If available, the molecular weight of the monomer was calculated from the derived amino acid sequence of the cloned gene, otherwise as determined by gel filtration* or SDS-PAGE+. [b] Type of enzyme; M Metallo-protease; S Serine-protease. [c] pH optimum of activity. [d] Localization as predicted from the absence of a signal sequence or membrane anchor sequence. [e] Localization as predicted from fractionation studies and immuno-gold labeling. [f] 50% inhibition by phenylmethylsulfonyl fluoride.

tides are hydrolysed (Hwang et al., 1981; Van Boven et al. 1988; Wohlrab & Bockelmann, 1992; Vongerichten et al. 1994; Tan et al., 1995). Of the 400 possible dipeptides about 80 have been tested, but only a minor fraction has been used for systematic studies. Comparison is therefore difficult, but again some generalizations can be made. Some preference towards N-terminal hydrophobic residues has been observed, whereas specificity for Pro-, Glu- or Asp-containing dipeptides is absent or very low (Van Boven et al., 1988; Tan et al., 1995). Small differences in specificity between PepVs of different organisms have also been noticed; the lactococcal dipeptidase hydrolyses Ala-Ala and Ala-Gly, whereas the dipeptidase of *Lb. helveticus* does not; the reverse is true for His-Leu (Tan et al., 1995).

Peptidases involved in the hydrolysis of Pro-containing peptides

Specialized peptidases capable of hydrolysing Pro-containing sequences have been postulated to be important for the degradation of casein-derived peptides because of the high content of proline in these molecules. General peptidases, such as PepN, PepC, and PepT, are also able to cleave Pro-containing tri- and oligopeptides *in vitro*, but activities observed are usually low (see above). To our knowledge, no significant hydrolysing activities have been found for Pro-containing dipeptides other than that of prolinases and prolidases (Table 4). By definition, the only difference between the specificity of a prolidase and aminopeptidase P, and between proline iminopeptidase and prolinase is the size of the substrate hydrolysed. Although distinct in substrate size limits, the biochemical and genetic properties of the enzymes are very similar (Table 4). Therefore, a thorough investigation of the upper substrate size-limits is required to correctly classify these enzymes.

Aminopeptidase P and prolidase. One aminopeptidase P (PepP) (Mars & Monnet, 1995) and two prolidases (PepQ) (Booth et al., 1990b; Kaminogawa et al., 1984) have been identified in different *L. lactis* subsp. *cremoris* strains. Recently, the gene encoding a prolidase (PepQ) from *Lb. delbrückii* subsp. *lactis* has been cloned and sequenced, and the deduced polypeptide was found to be homologous to PepP and PepQ of *E. coli* (Stucky et al., 1995b). PepP and PepQ are both monomeric metallo-peptidases of about 42 kDa. In agreement with its classification, PepP exclusively hydrolyses oligopeptides of up to 10 residues containing X↓Pro-Pro-$(X)_n$ or X↓Pro-$(X)_n$ sequences at the N-terminus (Mars & Monnet, 1995). No hydrolysis of di- and tripeptides with similar sequences occurs. The specificities of the three putative prolidases have been studied, but the upper size limits have not been systematically explored. One prolidase exclusively hydrolyses X-Pro dipeptides (Kaminogawa et al., 1984), while the other two also cleave dipeptides and tripeptides that do not even contain Pro (Booth et al. 1990b; Stucky et al., 1995b). The latter two enzymes also cleave Pro-X dipeptides, which is a typical activity for a prolinase.

Proline iminopeptidase and prolinase. By definition, a proline iminopeptidase recognizes tri- and oligopeptides which contain Pro-X sequences at the N-terminus, while a prolinase only cleaves Pro-X dipeptides. The proline iminopeptidases of four organisms have been characterized, and designated PepI (or PepIP) (Baankreis & Exterkate, 1991; Klein et al., 1994b; Atlan et al., 1994; Gilbert et al., 1994; Varmanen et al., 1996a). The primary sequence of PepI of *Lb. helveticus* shares 75% identity with that of *Lb. delbrückii* subspecies *lactis* and *bulgaricus* (Klein et al. 1994b; Atlan et al., 1994; Varmanen et al., 1996a). Two proline iminopeptidases are proteases with a subunit size of about 33 kDa, with the possible exception of the one characterized by Baankreis & Exterkate (1991) which is 50 kDa based on SDS-PAGE (or 36 kDa based on 110 kDa by gel-filtration and assumption of a trimeric quaternary structure). Sequence comparisons have revealed a structural motif GQSWGG indicative of a serine-active site, and inhibitor studies confirmed that the enzyme is a (metal-independent) serine peptidase (Atlan et al., 1994; Gilbert et al., 1994; Klein et al., 1994b). The lactococcal enzyme is the only proline iminopeptidase, which has been characterized as a metal-dependent enzyme (Baankreis & Exterkate, 1991). In view of the high similarity/identity of the primary sequences, it is puzzling that the *Lb. helveticus* enzyme is described as a dimer (Varmanen et al., 1996), while the *Lb. delbrückii* enzyme is characterized to be a trimer (Gilbert et al., 1994). The proline iminopeptidase of *Lb. delbrückii* subsp. *bulgaricus* hydrolyses preferentially Pro-X dipeptides and Pro-Gly-Gly, but also cleaves some Ala-X, Gly-X and Leu-X sequences, albeit with lower activities (Gilbert et al., 1994). Similar observations were made for the proline iminopeptidase of *L. lactis* subsp. *cremoris*. Two pentapeptides with Pro in first position were not hydrolysed, while a tetrapeptide with Pro in first position was degraded

with low activity (Baankreis & Exterkate, 1991). However, the upper size limits of the proline iminopeptidases have not been explored systematically.

Recently, a prolinase (PepR) has been found in two different strains of *Lb. helveticus*, and its gene has been cloned and sequenced (Dudley & Steele, 1994; Varmanen et al., 1996b). The amino acid sequence bears significant similarity with PepI of *Lb. delbrückii* (35%). The prolinase of *Lb. helveticus* has a subunit size of 35 kDa, which is similar to that of PepI and the same signature sequence (GQSWGG) is found. In agreement with its denomination, PepR predominantly hydrolyses Pro-X dipeptides, but also shows some activity towards tripeptides and Pro-lacking dipeptides.

X-prolyl-dipeptidyl aminopeptidase. Of all peptidases able to cleave Pro-containing sequences, the X-prolyl-dipeptidyl aminopeptidase (PepX) has received most attention. The PepX enzymes have been characterized as serine-peptidases. A number of *pepX* genes have been cloned from different dairy lactic acid bacteria (Mayo et al., 1991; Nardi et al., 1991; Meyer-Barton et al., 1993; Vesanto et al., 1995a, b; Yüksel & Steele, 1995). The primary sequence of PepX from *Lb. helveticus* is 70%, 37% and 37% identical to the enzymes of *Lb. delbrückii* subsp. *lactis*, *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis*, respectively. Some variations in the molecular mass of the monomer and the quaternary structure have been reported, but these differences may largely be due to the methods chosen for size determination (Table 4).

The substrate specificity of PepX has mainly been inferred from chromogenic substrates as X-Pro-*p*-nitroanilides or X-Pro-aminomethylcoumarins. Usually, PepX cleaves N-terminal X-Pro dieptides from tri- and oligopeptides (up to eleven residues) as, for instance, can be inferred from its activity towards Tyr-Pro-Phe, Tyr-Pro-Phe-Pro and β-casein fragment 60–66, Tyr-Pro-Phe-Pro-Gly-Pro-Ile (Kiefer-Partsch et al., 1989; Booth et al., 1990a; Lloyd & Pritchard, 1991; Yan et al. 1992; Sasaki et al., 1996b). The specificity is, however, not limited to X-Pro containing sequences, because Lys-Ala↓Val-Pro↓Tyr-Pro↓Gln (β-casein fragment 176–182) (Lloyd & Pritchard, 1991), and Tyr-Gly↓Gly-Phe-Met (Mayo et al., 1993) are cleaved at positions indicated by the arrows. On the other hand, Arg-Lys-Asp-Val, Arg-Tyr-Leu-Gly-Tyr-Leu and generally X-Pro-Pro-$(X)_n$ sequences are not hydrolysed (Booth et al., 1990a; Lloyd & Pritchard, 1991; Sasaki et al., 1996b).

Unique aminopeptidases

In addition to PepP, peptidase 53 and PepT of *L. lactis*, and PepD and PepR of *Lb. helveticus*, a number of other aminopeptidases have been identified, which have no counterpart in other organisms sofar.

Glutamyl aminopeptidase. In *L. lactis* subspecies, a glutamyl aminopeptidase (PepA) has been identified, which liberates N-terminal Glu and Asp residues from di-, tripeptides and oligopeptides consisting of up to ten amino acid residues (Exterkate & De Veer, 1987c; Niven, 1991; Bacon et al. 1994). This enzyme might well complement the inability of dipeptidases or other enzymes to release N-terminal acidic residues. Because glutamate is abundantly present as free amino acid in milk, PepA is probably not important for the liberation of Glu *per se*, but for the continued degradation of the peptides by other enzymes. The gene encoding this enzyme has been cloned and sequenced (l'Anson et al., 1995); and the deduced primary sequence is found to be homologous to endoglucanase from *Clostridium thermocellum* (30% identity). PepA is a metallopeptidase with a subunit size of 38 kDa and probably has a hexameric quarternary structure (Niven, 1991; Bacon et al., 1994), although a trimer has also been reported (Exterkate & De Veer, 1987c).

Pyrrolidone carboxylyl peptidase. The pyrrolidone carboxylyl peptidase (Pcp), identified in *L. lactis* subsp. *cremoris*, specifically cleaves N-terminal pyrrolidone carboxylyl residues of peptides and proteins (Exterkate, 1977). The primary sequence encodes a protein of 25 kDa, which shows significant similarity to analogous enzymes from *B. subtilis* and *Streptococcus pneumonia* (Haandrikman, unpublished results).

Leucyl aminopeptidase. Recently, the gene specifying a leucyl aminopeptidase (PepL) from *Lb. delbrückii* subsp. *lactis* has been cloned and sequenced (Klein et al., 1995). The gene product is predicted to be 35 kDa and can be classified as a serine-peptidase. This enzyme hydrolyses preferentially N-terminal Leu-containing dipeptides and some tripeptides. Another distinct aminopeptidase has been identified in *Lb. delbrückii* subsp. *bulgaricus* (Wohlrab & Bockelmann, 1994). It consists of eight subunits of about 32 kDa and might resemble the AC1-aminopeptidase identified in *L. lactis* subsp. *cremoris* (Geis et al., 1985). The enzyme is a metallo-enzyme and hydrolyzes di- and tripeptides and possibly also tetrapeptides. The high-

est hydrolysis rates were obtained with peptides carrying a N-terminal Leu or Lys, but activities towards Pro-containing di- and tripeptides were also measured.

Endopeptidases

Two different endopeptidases have been identified in *L. lactis* subsp. *cremoris*. One is a 70 kDa monomeric metallopeptidase capable of hydrolysing oligopeptides, but unable to hydrolyse caseins (PepO) (Tan et al., 1991). The gene encoding this enzyme has been cloned and sequenced, and the gene product exhibits significant identity to mammalian enkephalinases (27% amino acid identity) (Mierau et al., 1993). Study of the primary sequence revealed the presence of a consensus His-Glu-X-X-His sequence, typical for Zn-dependent metallopeptidases. Interestingly, the *pepO* gene is located immediately downstream of the genes of the oligopeptide transport system (*oppDFBCA*) (see above) (Tynkkynen et al., 1993). Following gene inactivation of *pepO*, another enzyme with similar activity, molecular mass and immunological properties was identified in this organism (Hellendoorn & Mierau, 1996). Cloning and nucleotide sequencing of this gene revealed that the amino acid sequence of PepO2 is 88% identical to that of PepO.

An additional endopeptidase activity with a specificity that is distinct from that of the PepO enzymes was purified by Monnet et al. (1994). This oligopeptidase was designated PepF; the *pepF* gene was cloned and sequenced, and was shown to specify a 70 kDa monomeric metallopeptidase. Sequence data show that this enzyme is different from the PepO enzymes, and that it resembles mammalian thimet oligopeptidases. Also this enzyme contains a typical Zn-binding site characteristic for Zn-dependent metallopeptidases. PepF hydrolyses peptides containing between 7 and 17 amino acids with a rather broad specificity. In contrast to PepO enzymes, this peptidase cannot degrade Met-enkephalin. Recently, another copy of the gene (80% identity) was found on a plasmid, which also carries the lactose utilization and proteinase genes, and the corresponding enzyme was designated PepF2 (Nardi et al., 1995). Similar enzymes as the ones described above have been purified from other strains of *L. lactis* (Muset et al., 1989; Baankreis 1992; Pritchard et al., 1994).

Recently, an endopeptidase of *Lb. helveticus* has been purified to homogeneity (Sasaki et al., 1996c). Like its lactococcal counterparts, it is a 70 kDa monomeric metallo-enzyme. The *Lb. helveticus* enzyme has a broad specificity for peptides of 3 up to 34 amino acid residues. It remains to be established whether this enzyme is PepO or PepF-like, but immunoblotting showed that it is immunologically distinct from the lactococcal enzymes. The genes coding for two endopeptidases have been cloned from another strain of *Lb. helveticus* (Fenster et al., 1996). One has a high amino acid identity to PepO of *L. lactis*, about the same size, and also contains a typical Zn-peptidase motif. The genes adjacent to this gene are not coding for the oligopeptide transport system like in *L. lactis*. The other putative endopeptidase is smaller (about 440 amino acids) and the primary sequence bears a cysteine peptidase motif. Protein homology searches revealed about 40% amino acid identity to PepC enzymes of lactic acid bacteria. This enzyme has been purified to homogeneity and appears to be able to hydrolyse Met-enkephalin in a PepO-like manner.

Carboxypeptidases

Until now no carboxypeptidases have been characterized in lactic acid bacteria.

Localization of peptidases

While the cellular location of the proteinase is undisputed, the localization of peptidases has been subject to controversies. Fractionation studies have suggested that some peptidases are present in cell-wall fractions, and on the basis of the assumption that PrtP-generated casein degradation products are too big for transport, extracellular peptidases have been implicated in the proteolytic pathway (Geis et al., 1985; Kiefer-Partsch et al., 1989; Exterkate & De Veer, 1987c; Blanc et al., 1993; Gilbert et al., 1994; Law, 1979; Law & Kolstad, 1983; Smid et al., 1989). Most biochemical, genetic and immunological data, however, suggest an intracellular location for most, if not all, enzymes studied to date (Tables 3 and 4). Below, the arguments and methods are listed that have been used to assign a cellular location to the peptidases. To our opinion, the experimental data described in the literature are often misinterpreted.

First, in most enzyme purification schemes, total cell extracts are used as starting material. Very rarely, cell fractionation of some sort is carried out as a first purification step (Sahlstrøm et al., 1993; Blanc et al., 1993; Gilbert et al., 1994), and only in isolated instances peptidase activity has been recovered from the growth medium (Law, 1977). For none of the

enzymes isolated and purified, detergents were used (or needed) to solubilize the proteins from the membrane. In all cases the purification methodology indicates that the isolated enzymes are readily soluble in water.

Second, the pH optimum of the enzyme may indicate where the enzyme activity would be maximal, but this information provides no argument for a possible cellular location.

Third, cell-fractionation studies are only useful when performed carefully and with the appropriate controls. For example, on the basis of fractionation studies the localization of the X-prolyl dipeptidyl aminopeptidase of *L. lactis* subsp. *cremoris* has been reported to be cell-wall bound (Kiefer-Partsch et al., 1989), inner-membrane bound (Yan et al., 1992) and cytoplasmic (Booth et al., 1990a). Critical for a correct interpretation of the results is the use of marker enzymes, both negative and positive for a given cell fraction. Many authors have isolated putatively extracellular enzymes after washing the cells several times with water or slightly alkaline buffers, or after incubation with lysozyme (Law, 1977; Geis et al. 1985; Kiefer-Partsch et al., 1989; Miyakawa et al., 1992). Such incubations may lead to significant cell lysis and, particularly, when enzymes are present in high amounts and/or high activities, the cellular location is easily erroneously assigned. To complicate things even further, many strains contain autolysins which render the cells variably susceptible to lysis (Coolbear et al., 1994; Chapot-Chartier et al., 1994).

Various enzymes such as β-galactosidase (Blanc et al., 1993; Atlan et al., 1994), glucose-6-phosphate dehydrogenase (Gilbert et al., 1994), aldolase and malate dehydrogenase (Sahlstrøm et al., 1993) have been used as cytoplasmic markers, but high activities are often associated with membrane preparations (Foucaud and Poolman, 1992; Poolman et al., 1991; Kunji unpublished results). Such activities can only be removed by appropriate procedures and by further purification of membrane fractions by density gradient centrifugation. A gentle shock of osmotically stabilized protoplasts with distilled water (Blanc et al., 1993) is inappropriate to isolate peripheral (external) membrane associated proteins. Such a treatment combined with extensive centrifugation is far more likely causing lysis of protoplasts and subsequent release of cytoplasmic enzymes. The variations in the extent of lysis can be dramatic as in one and the same study under similar conditions, values ranging from 0.1 to 77% have been observed (Blanc et al., 1993), which prevents any conclusions to be made. The use of antibodies as marker for fractionation studies has also to be taken with caution. Titers of the antibodies used may differ considerably and quantification is complicated by non-linearity between signal and protein amounts.

Fourth, immunogold labeling experiments have been performed on whole cells after fixation by aldehydes and embedding in Lowicryl K_4M (polymerization is induced by UV at -35 °C). Ultrathin sections are incubated with specific antibodies and labeled with protein A gold particles (Tan et al., 1992). Electronmicrographs reveal subsequently the localization of the gold-particles. Eminent to success of such an approach is the fixation of whole cells prior to incubation with specific antibodies. Any disruption of the cell membrane prior to or during fixation might lead to loss of enzymes, which may even be 'captured' by an undisrupted cell-wall and lead to misinterpretation of the data.

Fifth, the deduced primary sequence of a gene can give information on the presence or absence of a signal sequence required for translocation of the protein by the general secretory machinery (Sec pathway) (Driessen, 1994). Since signal sequences are not found in the peptidases studied to date, many authors have suggested the existence of dedicated secretory systems involved in the secretion of peptidases without cleavable signal sequence (Tan et al., 1992; Sahlstrøm et al. 1993). Although such systems have been described (Driessen, 1994), there is yet no evidence for signal sequence independent excretion of peptidases in lactic acid bacteria.

Sixth, as an adaptation to the absence of an outer membrane, membrane-associated enzymes of Gram-positives have anchors which keep the proteins attached to the cell. Proteins, as diverse as the maturation protein (PrtM) (Haandrikman et al., 1991), the oligopeptide binding protein (OppA) (Tynkkynen et al., 1993) and the nisin immunity protein (NisI) (Kuipers et al., 1993), all contain a typical signal sequence and a consensus lipo-modification site (generally LAX$\downarrow$C) (Sankaran & Wu, 1994). The cysteine that follows the cleavage site is modified with three fatty acid tails that anchors the mature protein to the external surface of the cell. In fact, even after extraction with urea/cholate, 80% of total OppA is still attached to membrane vesicles of *L. lactis* (Detmers & Kunji, unpublished results). Alternatively, other extracellularly located enzymes, such as the proteinase (PrtP) (Kok & De Vos, 1994) and the nisin maturation protein (NisB) (Kuipers et al., 1993), contain a signal sequence typical for the translocation by

the Sec-dependent secretory pathway (Palmen et al., 1994) and a C-terminal anchor (see above) (Navarre & Schneewind, 1994). A putative anchor can be deduced from sequence comparisons, hydropathy profiling and secondary structure predictions.

Localization of individual peptidases in lactococci

Tables 3 and 4 list the general properties of most peptidases of dairy lactococci and lactobacilli characterized to date and their proposed localization. Assignment of the cellular localization is based on fractionation and immunogold labelling studies, and on the presence or absence of a membrane anchor and/or signal sequence as can be inferred from protein sequences. Listed is also the pH range in which the enzyme has optimum activity.

The genes of eleven peptidases of *L. lactis*, i.e. PepO, PepO2, PepF, PepF2, PepN, PepC, PepT, PepV, PepA, PepX and PcP, have been cloned and sequenced [most of them are reviewed in Kok & De Vos (1994) and Poolman et al. (1995); others are described in Monnet et al., 1994; l'Anson et al., 1995; Nardi et al., 1995; Hellendoorn & Mierau, 1996; & Faynard & Mierau 1996]. In none of the inferred amino acid sequences of the peptidase genes a typical signal sequence or membrane anchor is detected. Data obtained from fractionation and immunogold labelling studies point towards an intracellular localization for PepN, PepO, PepT, PepX and PepC (see Tables 3 and 4) or are conflicting, e.g. PepA (Exterkate & De Veer, 1987c; Bacon et al., 1994) and PepX (Kiefer-Partsch et al., 1989; Booth et al., 1990a; Yan et al., 1992). Sequence data are lacking for AC1-aminopeptidase (Geis et al., 1985), and peptidase 53 (Sahlstrøm et al., 1993).

The view that most, if not all, lactococcal peptidases are located inside the cell is also supported by growth and uptake experiments performed with isogenic peptide transport mutants. If a peptide transport mutant is unable to utilize a particular peptide, it must mean that peptidases involved in hydrolysis of the peptide are **not** located extracellularly, since extracellular breakdown products would otherwise have entered the cell via the amino acid and/or, in case of Opp substrates, via the di- and tripeptide transport systems. Substrates as diverse as Ala-Ala, Pro-Ala, Ala-Pro, Ala-Pro-Leu, Leu-Gly-Gly, Ala-Ala-Ala-Ala, Tyr-Gly-Gly-Phe-Leu and Val-His-Leu-Thr-Pro-Val-Gly-Lys are not hydrolysed extracellularly, providing compelling evidence for an intracellular location of PepV, PepR (sofar not characterized), PepQ, PepX, PepT, PepN, PepC, PepO and PepO2 (Tynkkynen et al., 1993; Hagting et al., 1994; Kunji et al., 1995; Foucaud et al., 1995; Kunji et al., 1996). Similar experiments with substrates typical for peptidase 53 (Sahlstrøm et al., 1993), such as Leu-Leu-Leu, have made it highly unlikely that indeed this enzyme is present extracellularly in *L. lactis* MG1363.

Moreover, in peptide transport assays in *L. lactis*, a wide variety of peptides (from 2 up to ten residues) have been used, and extracellular and intracellular fractions have been analysed by liquid chromatography (sometimes in combination with mass spectrometry) (Fang & Kunji, unpublished results). Under conditions that cell lysis is minimal, through the use of mutants in which an autolysin gene is inactivated (Buist et al., 1995), we have never detected any extracellular hydrolysis of peptides, not even when high cell densities were used. In fact, by comparing amino acid accumulation from β-casein degradation in wildtype cells and oligopeptide transport mutants, we could demonstrate that extracellular peptidase activity involved in degradation of this protein substrate is lacking in *L. lactis* (see below) (Kunji et al., 1995). In addition, recent studies, in which the product formation of the purified proteinase was compared to that of PrtP present on the cell surface of oligopeptide transport and autolysin deficient mutants, have shown that the casein-derived peptides are not significantly altered even after prolonged incubations (Kunji & Fang, unpublished results).

Localization of individual peptidases in lactobacilli

Sequence data and fractionation studies indicate that the majority of peptidases in lactobacilli is located in the cytoplasm (Tables 3 and 4), but conflicting data have also been reported. In *Lb. helveticus* two PepN-like aminopeptidases have been described to be cell-wall associated (Miyakawa et al., 1992; Blanc et al., 1993). Recent immunological studies have indicated that PepN as well as PepV, PepX and PepO of *Lb. helveticus* are located intracellularly (Bosman et al., 1996). In addition, the gene encoding PepN does not contain a signal sequence or membrane anchor (Christensen et al., 1995).

The prolyl iminopeptidase of *Lb. delbrückii* subsp. *bulgaricus* is sofar the only proline-specific peptidase which has been detected in cell-wall fractions (Gilbert et al., 1994), but the deduced amino acid sequence does not contain a signal sequence or membrane anchor (Atlan et al., 1994). The observed periplasmic location in *E. coli* of 45% of total prolyl iminopeptidase activ-

ity is probably an artifact of heterologous expression or isolation procedure (Gilbert et al., 1994). Hydropathy profiling of the deduced protein indicated that two short hydrophobic sequences are present, but this is not uncommon for interior stretches of soluble proteins.

Concluding remarks

In conclusion, it strikes as somewhat odd that peptidases, which are so similar in primary sequence and function, would be located at different places in different lactic acid bacteria. Consensus about the location for all peptidases of the same class would be expected, whether inside or outside. The localization of peptidases has also been studied in detail in *E. coli* and *S. typhimurium*. In these organisms, peptidases with very similar biochemical characteristics, and in many cases homologous to those of lactic acid bacteria, have all been found intracellularly (Lazdunski, 1989). Extracellular degradation of peptides down to amino acids would lead to a substantial loss of amino acids through diffusion. In contrast, amino acids liberated from intracellularly accumulated peptides, can directly be used for biosynthesis or other metabolic activities. In view of the concept that concerted action of peptidases is required for complete degradation of peptides, it seems logical that peptidases are located in the same compartment, otherwise, extensive relocation of degradation products would be necessary to complete hydrolysis. The degradation is most efficient when the concentration of enzymes and substrates is highest, which is most easily achieved when these are gathered in the cytoplasm. A prerequisite for efficient degradation by cytoplasmic peptidases is that the PrtP-generated casein breakdown products can be translocated into the cell.

The role of peptide transport systems in growth on milk

In recent years, a number of directional mutagenesis techniques have been developed for inactivation of chromosomally located genes (Leenhouts, 1991; Bhowmik et al., 1993; Leenhouts et al., 1996). Mutants have been constructed which lack a functional di- and tripeptide transport system (DtpT) and/or oligopeptide transport system (Opp), but do express the P_I-type proteinase (Tynkkynen et al., 1993; Hagting et al., 1994; Kunji et al. 1995). Mutants which lack a functional Opp system are unable to grow on milk (Tynkkynen et al., 1993), while growth of mutants lacking DtpT is unaffected in this medium (Kunji et al., 1995). This observation indicates that one or more essential amino acids enter the cell via uptake through Opp. To circumstantiate this finding, cells were incubated with β-casein in the presence of glucose and chloramphenicol. The wildtype strain and the $DtpT^-$ mutant accumulate all amino acids present in β-casein (Figure 4A), whereas amino acids are not accumulated significantly inside the cells of Opp^- and $DtpT^-$ Opp^- mutants (Figure 4B). When cells are incubated with a mixture of amino acids mimicking the composition of β-casein, the amino acids are taken up to the same extent in all four strains.

These and other experiments have revealed a number of important properties of the proteolytic pathway of *L. lactis*. First, all the essential and growth-stimulating amino acids can be released from β-casein by the action of the proteinase PrtP in a form that can be transported by the oligopeptide transport system exclusively. When a functional oligopeptide transport system is absent no significant intracellular accumulation of amino acids is observed. Second, consistent with the observation that PrtP does not release significant amounts of di- and tripeptides from β-casein (Juillard et al., 1995a), inactivation of the di-tripeptide transport system has no effect on the utilization of this protein substrate. Since di-tripeptide transport mutants selected on the basis of resistance towards L-Ala-β-chloro-L-Ala are affected in their ability to grow on a mixture of caseins (Smid et al., 1989), we speculate that this phenotype is due to secondary mutations or to the inability to transport essential amino acids (most likely His and/or Leu) in the form of small peptides that are released from proteins other than β-casein. In fact, growth of *L. lactis* HP has been shown to be dependent on the utilization of both β- and κ-casein (Exterkate & De Veer, 1987b). Third, the observation that a single mutation, abolishing oligopeptide transport activity, results in a defect to accumulate amino acids argues strongly against the involvement of extracellular peptidases in the degradation of β-casein. If peptidases would have been present externally, amino acids and di- and tripeptides would have been formed and subsequently been taken up by the corresponding transport systems.

Preliminary results show that peptides from the C-terminal end of β-casein are transported by Opp (indicated as dotted arrows in Figure 1A). Most of these peptides are in the range of 4 to 8 residues, but transport of at least one nonamer and one decamer has been observed. Transport of these peptides into the

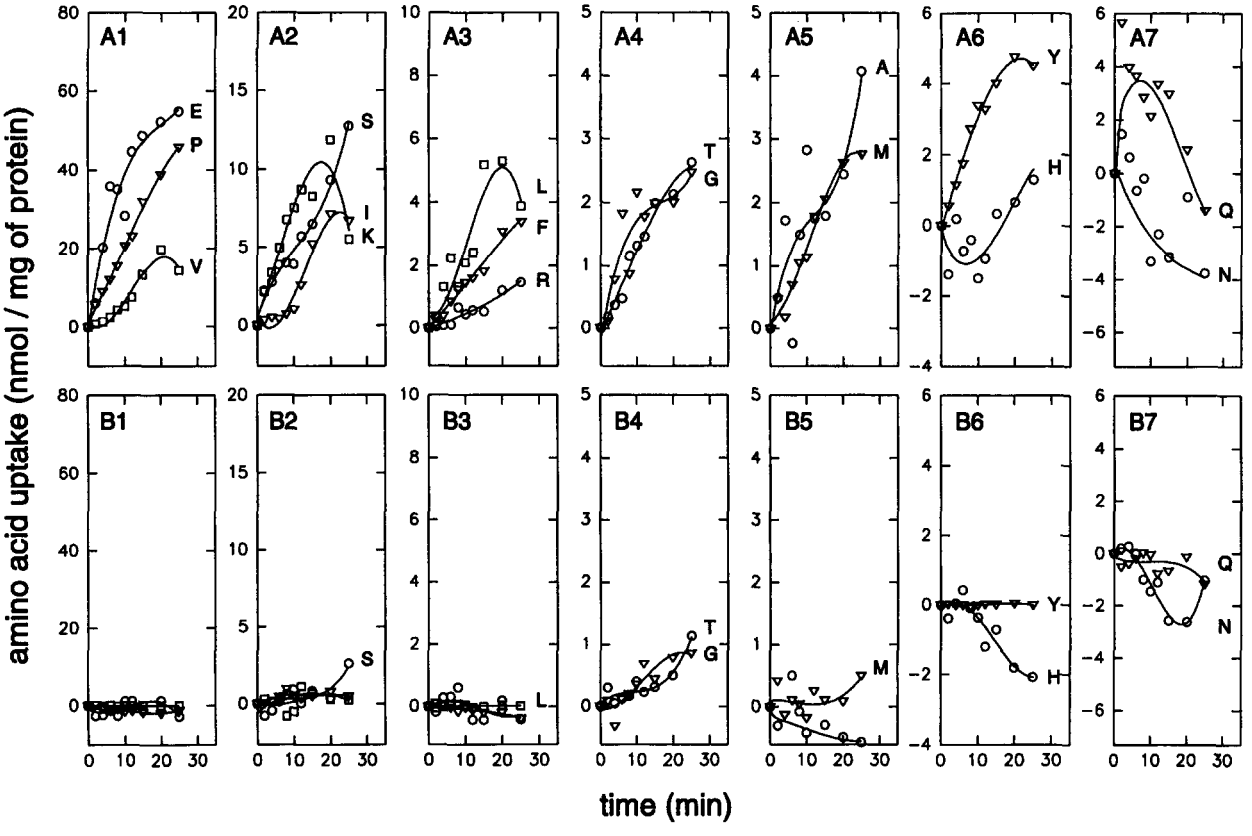

Figure 4. Time dependence of intracellular amino acid accumulation by *L. lactis* wildtype (A) and oligopeptide transport mutant (B). β-casein was added to glycolyzing chloramphenicol-treated cells at time zero (min). The amino acids are indicated by their 1 letter code. (Data from Kunji et al., 1995.)

cell largely explains the intracellular amino acid accumulation upon addition of β-casein to wildtype cells. Amino acids that are rare in β-casein, such as Ala and Tyr, are readily accumulated and these residues are indeed present in the C-terminal fragments. As stated before, the C-terminal peptides are flanked by preferential cleavage sites and are present in relatively large amounts in the hydrolysates, already at the earliest times of degradation. Transport of these peptides would supply the cell with all essential and growth stimulating amino acids with the exception of His. Even though Glu and Asn are not present in these fragments, Gln and Asp can be converted into these amino acids. Growth experiments with β-casein have shown that His and Leu are not liberated from β-casein at rates high enough to meet the growth requirements of *L. lactis* (Kunji et al., 1995). In hydrolysates of κ-casein, peptides are present which fall within the size exclusion limits of Opp and contain His and Leu. The degradation of α-caseins may also contribute significantly to supply of amino acids such as Leu, i.e. when the appropriate proteinase activity is present.

The role of peptidases in vivo

In recent years a number of single peptidase mutants have been constructed by targeted deletion or disruption of the corresponding genes. Lactococcal mutants lacking either PepX, PepN, PepO, PepT, PepF, PepC and PepA are not or only slightly affected in their ability to grow in milk (Mayo et al., 1993; Baankreis, 1992; Mierau et al., 1993; Mierau et al., 1994; Monnet et al., 1994; Erra-Pujada et al. 1995; l'Anson et al., 1995). Similar results have been obtained when single mutations of PepC, PepN and PepX were generated in *Lb. helveticus* (Christensen and Steele, 1996). The observation that single peptidases are not essential can have several reasons: (i) some peptidases might not be involved in casein degradation at all, (ii) peptidases with overlapping specificities might be present and/or

(iii) alternative peptides, whose degradation is undisturbed by the mutation, might be used to supply the cell with free amino acids.

Recently, a set of sixteen single and multiple peptidase deletion mutants has been constructed in *L. lactis*, in which combinations of up to all five of the following peptidase genes were inactivated: *pepO, pepN, pepC, pepX* and *pepT* (Mierau et al., 1996). When the ability of these strains to grow in milk was tested, it was observed that an increasing number of peptidase mutations leads to an increasing growth defect (Mierau et al., 1996) (Figure 5). Ultimately, the fivefold mutant grows more than 10 times slower in milk than the wild-type strain. Similar results were obtained with *Lb. helveticus* mutants, in which combinations of *pepC, pepN* and *pepX* were made (Christensen & Steele, 1996). The main exception being that PepX appears to play a more important role in this organism than in *L. lactis*.

In *L. lactis*, the growth of the manifold mutants in complex broth was identical to that of the wildtype, indicating that the lower growth rates in milk are not due to a general defect in proteolytic housekeeping functions of the cell. The phenotype of the mutants is also not caused by a decreased expression or activity of the other components of the proteolytic pathway, since neither PrtP, Opp nor other peptidase activities were seriously affected by the peptidase mutations (see below).

The slowest growing mutants, i.e. $[XTNC]^-$ and [XTOCN], had much lower intracellular amino acid pools than the wild-type, and peptides were accumulated inside the cell of these mutants. Thus, the lower growth rates can directly be attributed to the inability of the mutants to breakdown casein-derived peptides, providing, for the first time, direct evidence for the functioning of lactococcal peptidases in the degradation of milk proteins. The observation that the five-fold mutant has growth rates close to zero, also indicates that PepN, PepC, PepO, PepT and (to a lesser extent) PepX are crucial for the degradation of casein-derived peptides.

The complexity of the peptide mixture in milk makes it difficult to trace the fate of individual molecules and to assign a particular role to individual peptidases in the proteolytic pathway. Therefore, the same set of peptidase mutants of *L. lactis* has been used to study the fate of single peptides in growth experiments and by chromatographic analysis of intracellular fractions (Kunji et al., 1996). Several multiple peptidase mutants were unable to utilize particular peptides and, as a consequence, accumulated these peptides intracellularly. Apparently, the failure to grow relates to the inability of the cells to hydrolyse the peptides. The observation that peptide transport mutants are impaired in their ability to accumulate peptides, combined with the finding that peptides are translocated into the cell as whole entities, proves unequivocally that transport precedes degradation (Kunji et al., 1996).

Mutants lacking PepN, PepC, PepT plus PepX cannot utilize peptides such as Leu-Gly-Gly, Gly-Phe-Leu, Leu-Gly-Pro, Ala-Pro-Leu and Gly-Leu-Gly-Leu, indicating that no other peptidases are present in *L. lactis* MG1363 to hydrolyse these molecules. The fivefold mutant $[XTOCN]^-$ still grows on Gly-Leu and Tyr-Gly-Gly-Phe-Leu, confirming the presence of a dipeptidase and another endopeptidase (e.g. PepO2). The general aminopeptidases PepN, PepC and PepT have overlapping, but not identical specificities and differ in their overall activity towards individual peptides (Kunji et al., 1996). In contrast, PepX has a unique specificity, because it is the only enzyme which degrades Ala-Pro-Leu efficiently. Certain peptides can only be broken down by the concerted action of different peptidases, e.g. in a *pepN* background, Leu can only be liberated from Gly-Leu-Gly-Leu if PepC plus PepT are present.

Regulation of expression of components of the proteolytic pathway

The regulation of expression of the various components of the proteolytic pathway is a still largely unexplored area of research. Few studies have been performed in which the expression of enzymes has been studied in different media, including whey permeate, milk, complex media, and chemically defined media (CDM) with either amino acids, peptides or caseins. Published data cannot always be compared directly due to differences in strains, variations in media, etc. In general, the expression of components of the proteolytic pathway of *L. lactis* is highest in media containing amino acids only, while peptides generally down-regulate expression.

In *L. lactis* AM1, synthesis of the proteinase is repressed when the growth medium contains casitone, an enzymatic digest of casein that mainly consists of peptides (Exterkate, 1985). The production of the proteinase of *L. lactis* Wg2 is also inhibited in media containing casein or a tryptic digest of casein (Laan et al., 1993). Moreover, the addition of the dipeptide Leu-Pro to chemically defined medium leads to a decrease of the proteinase production by this strain (Laan et al.,

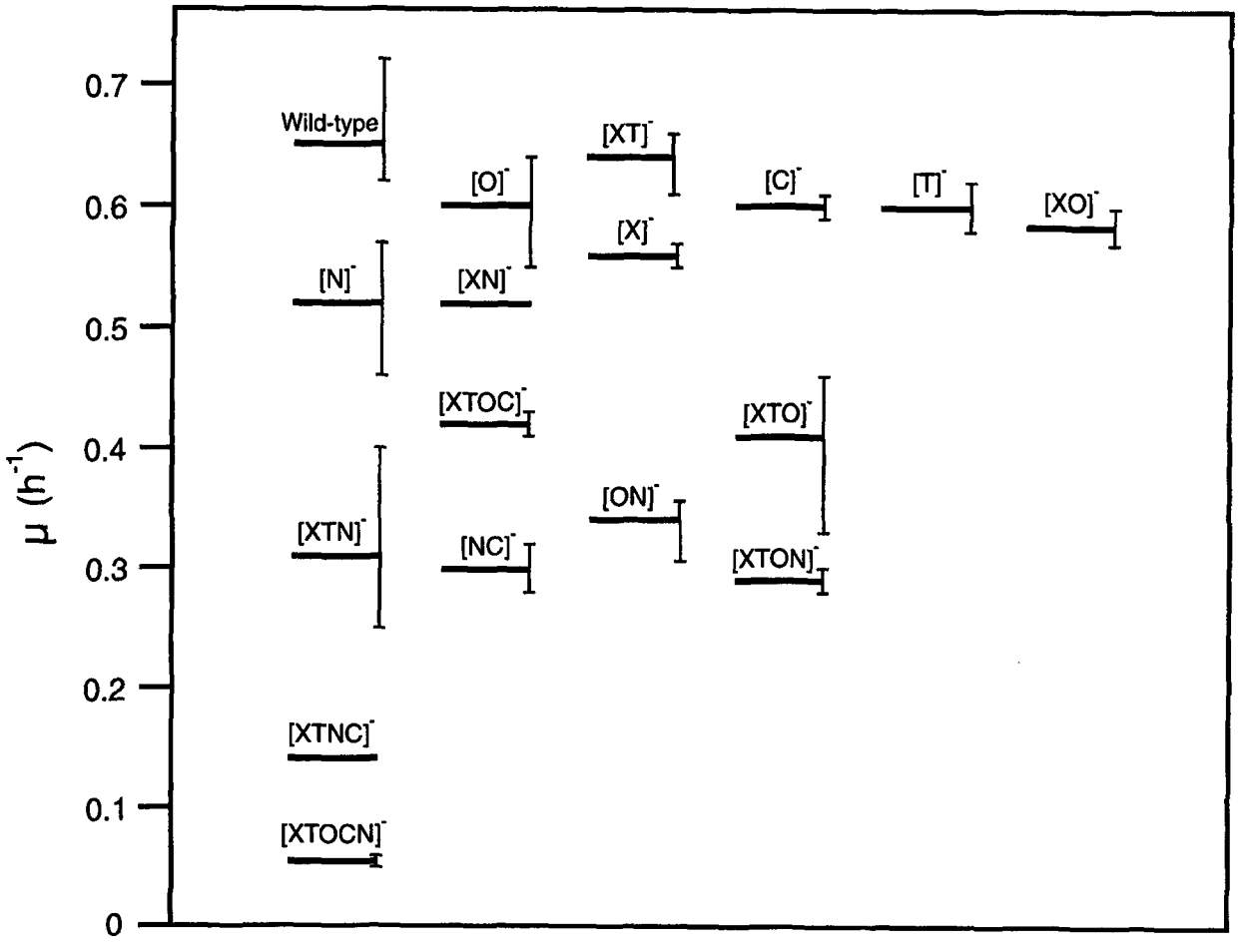

Figure 5. Growth rates of *Lactococcus lactis* MG1363 and peptidase mutants in milk. Horizontal bars indicate the growth rates and vertical bars mark the variation of the data by showing the range of the highest and the lowest values measured for a particular strain. X, T, O, C and N refer to deletions of *pepX, pepT, pepO, pepC* and *pepN* genes, respectively. (Data from Mierau et al., 1996.)

1993). By fusing the promoter regions of *prtP* and *prtM* to the reporter gene *gusA*, it was possible to follow the transcriptional regulation of the proteinase expression directly (Marugg et al., 1995). The GusA activities were highest in media containing no or low amounts of peptides, and repression is observed in peptide-rich media. Again, addition of Leu-Pro and Pro-Leu were found to down-regulate the expression of PrtP in wild-type cells. Importantly, in mutants, lacking DtpT, the synthesis of GusA (driven by the PrtP and PrtM promoters) was not affected by these dipeptides. In agreement with these results, significant higher expression of PrtP was observed when mutants lacking DtpT were grown in a complex medium as compared to the wild-type (Kunji et al., 1995).

Also the expression of the peptide transport systems of *L. lactis* is (moderately) affected by the composition of the growth medium. Expression of DtpT is five-fold higher when cells are grown on chemically defined media containing only amino acids as compared to complex media containing both peptides and amino acids as source of nitrogen (Hagting, unpublished results). Likewise, the expression of OppA is increased more than 10-fold when cells are grown on chemically defined media containing amino acids rather than complex broth (Detmers and Kunji, unpublished results). The expression of DtpP, increases two to three-fold when CDM is used instead of a complex medium to culture the cells (Foucaud et al., 1995). A similar increase in transport activity is observed when Leu-Leu or Leu-Leu-Leu are added to CDM, suggest-

ing that, in contrast to DtpT and Opp, these peptides may serve as inducers of the DtpP system.

The regulation of peptidase expression has not been studied in detail. Preliminary experiments suggest that expression of PepN in *L. lactis* is regulated in a similar manner as the proteinase (Meijer et al., 1995). Genes specifying putative regulator proteins have been found in the proximity of peptidase genes. A gene coding for a potential transcription regulator protein, designated PepR1, was identified by sequence comparisons and is located upstream of the prolidase gene *pepQ* of *Lb. delbrückii* subsp. *lactis* (Stucky et al., 1996). The deduced protein has a molecular mass of 37 kDa and shows significant similarity to catabolite control proteins of various organisms (Stucky et al., 1996). A hybrid of *pepQ* and the β-galactosidase reporter gene displays an enhanced expression when co-expressed with *pepR1* in *E. coli*.

Despite the observations that synthesis of components of the proteolytic system of *L. lactis* is affected by peptides, no major changes were observed in the specific activities of PrtP, Opp and the remaining peptidases in the peptidase mutants when the cells were grown in milk (Mierau et al., 1996). This is surprising, because one would expect that under the nitrogen starvation conditions prevailing in the multiple peptidase mutants, the cells would want to compensate for the peptidase deficiency by increasing the expression levels of other components of the proteolytic system. Thus, the regulation of expression through changes in amino acid and/or peptide levels in the cell appear to be minimal when *L. lactis* is grown in milk.

The proteolytic pathway of Lactococcus lactis*: a model*

Gene inactivation experiments have demonstrated that PrtP and Opp are essential for growth of *L. lactis* on media containing casein(s) as sole source of amino acids (Kok & De Vos, 1994; Tynkkynen et al., 1993; Kunji et al., 1995; Juillard et al., 1995b). Various lines of evidence indicate that the peptidases are located intracellularly and, consequently, cannot play a role in extracellular hydrolysis of caseins or casein-derived peptides (unless cell lysis occurs). These observations, together with the evidence that the Opp system transports peptides with a length of 4 up to at least 8 residues (maybe 10), suggest that a major fraction of essential and growth-stimulating amino acids enters the cell in the form of oligopeptides (Figure 6). A large number of peptides are released from β-casein by the activity of the proteinase, and a major fraction of the β-casein derived peptides falls in the range of 4 to 10 residues (Juillard et al., 1995a). Several of these peptides are released from the C-terminal part of β-casein in high amounts, irrespective of the type of proteinase (Figure 1A). Figure 1B shows the identified peptides released from activity of the various proteinases on β-casein. The β- and κ-casein-derived oligopeptides together contain all amino acids necessary for growth of *L. lactis*.

From the analysis of the extracellular and the intracellular fractions it can be concluded that only a few of the peptides generated by the proteinase are actually utilized by *L. lactis* (Kunji et al., 1995; Fang & Kunji unpublished results). The fact that large peptides do accumulate in the medium despite a functional Opp system is a consequence of the size-exclusion limits of the oligopeptide transporter. Peptides up to a length of 30 amino acids are formed by PrtP, and, although the upper size exclusion limits of Opp have not been established unequivocally, most of these large fragments will not be transported (Juillard et al., 1995a). Furthermore, although the lactococcal oligopeptide transport system has a broad substrate specificity, certain peptides may not be transported due to competition of peptides for the oligopeptide binding protein. In addition, a part of the peptide pool may also be taken up with a rate that is lower than the production rate by the proteinase.

Since transport precedes degradation (see above), the specificity of the Opp system will largely determine the oligopeptides which enter the peptidolytic pathway during growth in milk. These peptides are degraded efficiently by a multitude of peptidases, because all peptidases which are needed for degradation are present in the cytoplasm (Figure 6).

The peptidases can be divided into two classes: (1) *primary peptidases*, that generate free amino acids from oligopeptides directly (PepN, PepA, PepI, PepP and possibly PepC), and (ii) *secondary peptidases*, that need degradation by other peptidases to complete hydrolysis to the level of free amino acids (PepO, PepO2, PepF, PepF2, PepX, PepR, PepQ, PepT and PepV) (Figure 6). Endopeptidases (and PepX) require further degradation of the breakdown products by aminopeptidases, tri- and dipeptidases, while di- and tripeptidases require the initial activity of oligo- and/or aminopeptidases capable of hydrolysing oligopeptides. From inactivation studies it has become apparent that PepN, PepC, PepT, PepX and PepO activities are crucial in the peptidolytic pathway. Inactivation of the

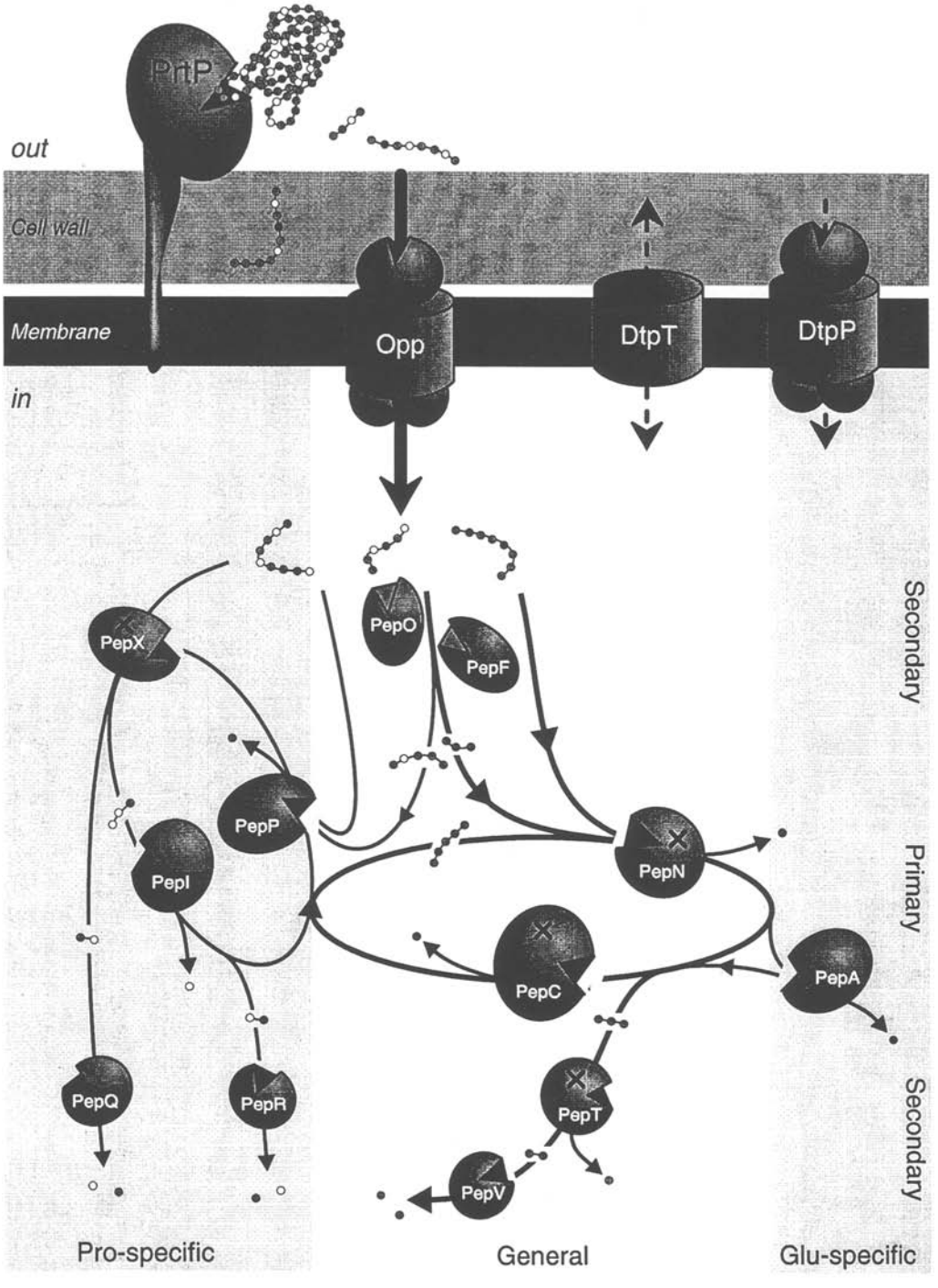

Figure 6. Proposed model for the proteolytic system of *Lactococcus lactis*. The role of the *primary* (PepN, PepC, PepI, PepP and PepA) and *secondary* enzymes (PepO, PepF, PepX, PepQ, PepR, PepV and PepT) and peptidolytic cycles is depicted schematically (various alternative routes of breakdown are possible for most peptides). For simplicity, PepO2 and PepF2 are not depicted. The peptidases inactivated in the five-fold mutant (see text) are indicated by X. Proline residues of casein or casein-derived peptides are depicted as white circles and Glu/Asp as black.

primary peptidases PepN and PepC will directly affect the release of amino acids from oligopeptides (Mierau et al., 1996; Christensen & Steele, 1996). The five-fold peptidase mutant has growth rates in milk close to zero, even though it still contains all the dipeptidases and endopeptidases (only PepO is missing), and several specific aminopeptidases (PepA, PepI and PepP). This indicates that degradation to the levels of dipeptides is severely impaired in this mutant and that the proteinase does not produce dipeptides in large amounts. Moreover, the five-fold mutant provides evidence for the notion that the endopeptidases, PepO2, PepF and PepF2, do not form significant amounts of dipeptides, and dipeptide formation by endopeptidases has thus become dependent on other activities which are inactivated in the multiple peptidase mutant. The Pro- and Glu/Asp-specific peptidases capable of oligopeptide degradation, i.e. PepI, PepP and PepA, are clearly not active enough and/or too specific to complement the peptidase deficiencies in the five-fold mutant.

Concluding remarks and prospects

The model proposed for *L. lactis* is compatible with most studies on specificity, location and role of the various enzymes in proteolysis. However, important questions remain to be answered. Are the casein degradation experiments *in vitro* a true reflection of the situation in milk? In the *in vitro* experiments, purified casein preparations are used, while in milk, caseins are in part organized in micelles. In addition, other proteolytic activities naturally present in milk, such as that of plasmins, might alter the product formation on caseins considerably and thereby influence the growth of lactic acid bacteria (Grufferty & Fox, 1988). Recently, the plasmin precursor plasminogen has been isolated from bovine milk and characterized (Benfeldt et al., 1994). Studies on the product formation of plasmins on caseins have been intiated in the laboratory of Petersen.

Does a purified enzyme yield the same product formation as a cell wall associated enzyme? Preliminary data indicate that peptides which are thought to be important for growth are released by the purified as well as the cell-wall associated enzymes (Fang & Kunji, unpublished results). How is casein-degradation influenced by the decreasing pH values prevailing during growth in milk? Does the degradation of casein by other proteinases indeed proceed as far as reported for the *L. lactis* Wg2 proteinase (Juillard et al., 1995a)?

Why are amino acid transport systems present in lactic acid bacteria? During initial growth of *L. lactis* in milk, amino acids are used as sources of nitrogen, but total consumption of free amino acids is very low (Juillard et al., 1995b). Peptide transport mutants, which are only able to utilize free amino acids, grow only to very low cell densities. Based on these studies it could be estimated that free amino acids contribute less than 2% to growth of the organism in milk. Alternatively, the amino acid transport systems might play a role in maintaining a balanced amino acid pool in the following way. The composition of transported oligopeptides will not correspond to the amino acid needs of the organism. If the concentration gradients of the amino acids exceed the driving force imposed by the amino acid transporter, the residues may leave the cell by facilitated diffusion. If efflux of amino acids via the transport proteins is coupled to proton extrusion, a proton motive force will be generated. In fact, if the total energy costs of oligopeptide uptake are lower than the amount of energy generated by amino acid residue driven efflux of protons, metabolic energy is generated. Such mechanisms might be important for the survival of lactic acid bacteria in dairy products when milk sugars have been depleted.

What is the function of DtpT and DtpP? Inactivation of the di/tripeptide transport systems does not result in a growth defect in milk (Kunji et al., 1995; Juillard unpublished results). Since DtpT is just like most amino acid transport systems a reversible enzyme, it is possible that DtpT can also function as an efflux system for partial degradation products.

What role has the putatively extracellular peptidase 53 in the proteolytic system (Sahlstrøm et al., 1993)? Our results indicate that mutations in *pepT, pepX, pepN* plus *pepC* remove all tripeptidase activity in *L. lactis* MG1363 (Kunji et al., 1996). What is the role of proline- and glutamate/aspartate-specific peptidases and of the PepF enzymes in the degradation of casein-derived peptides? It might well be that some of these peptidases also have roles in intrinsic protein turnover.

The model we propose for *L. lactis* may be extrapolated to the proteolytic systems of *Lactobacillus* species, but even more questions remain to be answered because several components of the proteolytic pathway of these bacteria have been studied in much less detail. Anyhow, as far as a comparison can be made, the proteolytic systems of lactococci and lactobacilli appear to be very similar. Since these organisms are faced with similar challenges during growth in milk, profound

differences in the general scheme of the proteolytic pathway are not to be expected. All organisms require efficient degradation of caseins for optimal growth. The amount of amino acids necessary for growth is strain dependent, but caseins provide enough amino acids to meet any demand. For initial degradation, most lactic acid bacteria possess a single extracellularly located proteinase, which degrades caseins into oligopeptides. These enzymes are subject to only little genetic variation. Since the casein-derived peptides are variable in composition and size, concerted action of peptidases is required to complete degradation and this is most efficiently done when all enzymes are located at the same location. Although peptide transport systems are still poorly characterized in lactobacilli, preliminary experiments suggest that activities similar to those in *L. lactis* are indeed present.

If the overall strategy of dairy lactic acid bacteria to grow in milk is the same, how is it possible that these organisms yield such a variety of different dairy products? A recent comparison of proteolytic activities of different lactococci and lactobacilli has revealed that overall activities of enzymes may vary considerably (Crow et al., 1994; Sasaki et al., 1995). Such variations are not important for growth of the organism (the amounts of amino acids produced from caseins are always in excess), but might have a profound effect on flavour and texture of the final food product. Additionally, through acquired mutations, the enzymes might have an altered sensitivity towards pH values, salt concentrations, temperature and prolonged periods of incubation, which are conditions prevailing during ripening of dairy products. In addition to differences in activity of the enzymes, particular activities seem to be confined to certain *Lactobacillus* and *Lactococcus* strains. For instance, significant PepA-like activity has only been detected in lactococci and not in lactobacilli (Sasaki et al., 1995).

Finally, since the peptidases are located intracellularly, differences in susceptibility to lysis might play an important role in ripening of dairy products (Chapot-Chartier et al., 1994; Coolbear et al., 1994; Buist et al., 1995) and offers a means to manipulate proteolysis.

The proteolytic system of lactic acid bacteria has become the paradigm of research on proteolysis in bacteria. With the genetic and biochemical tools available, it has now become possible to manipulate the pathways of protein and peptide degradation, and amino acid and peptide transport. These developments have paved the way to new, more economical and better quality food products.

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