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Casein and Peptide Degradation in Lactic Acid Bacteria

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Introduction

Lactococcus lactis is an important component of many starter cultures used in cheese manufacturing (*Table 1*). The function of starters is the production of lactic acid from lactose, the degradation of casein and, in some instances, the production of antimicrobial agents. In this way they contribute to optimal curd formation, to the exclusion of undesired spoilage bacteria and to the development of the desired texture and flavour of the cheese (Hoover and Steenson, 1993; Kok and de Vos, 1994; Nath, 1992). Modern industrial large-scale cheese production with a throughput of up to one million litres of milk per day has dramatically increased the demands for a reliable and stable performance of the starter cultures and has inspired a thorough microbiological, biochemical and genetic investigation of *L. lactis*. Key targets of research are: the proteolytic system, carbohydrate metabolism, bacteriophages and bacteriophage resistance, bacteriocin production and mechanisms of bacteriocin action, and the exploration of new applications of starter culture bacteria, such as live vaccine development (Davis and Law, 1984; Gasson and de Vos, 1994; Gottschalk, 1993; van de Guchte *et al.*, 1992). The present review will focus on the proteolytic system of the model lactic acid bacterium *L. lactis* and its role in the growth of the organism in milk and during cheese ripening. Furthermore, a brief overview will be given on the rapidly accumulating knowledge on the proteolytic systems of other important starter lactic acid bacteria.

Rapid growth of *L. lactis* in milk with the concomitant production of lactic acid is essential in the early phases of cheese making. The organism lacks the ability to

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synthesise several amino acids and, therefore, depends on the supply of the necessary amino acids from the medium (Chopin, 1993). The amount of small peptides and amino acids in milk is limited and only allows growth to final cell densities of 5%–25% of a full grown culture. Milk protein (casein) is the major source of amino acids for growth to high cell densities (Julliard *et al.*, 1995b; Thomas and Pritchard, 1987). Therefore, one of the main functions of the proteolytic system of *L. lactis* (*Figure 1*) is to make the amino acids present in casein available for growth. This is accomplished in a three-step process. First, the extracellular proteinase (PrtP) degrades casein into peptides. Second, several of these peptides are incorporated by the cell via an oligopeptide transport system (Opp). The third and last step is the degradation by peptidases of these peptides into amino acids which are then available for *de novo* protein synthesis and other metabolic activities. During cheese ripening both PrtP and the peptidases play a role in texture development and flavour formation (*Figure 1*) (Kok and de Vos, 1994; Poolman *et al.*, 1995; Visser, 1992).

Table 1. Cheeses and their starter cultures (adapted from Nath, 1992)

Cheese	Composition of the starter culture
Cheddar	*Lactococcus lactis* subsp. *lactis* and subsp. *cremoris*, (optional: *Leuconostoc mesenteroides* subsp. *cremoris* and *L. lactis* subsp. *lactis* biovar. *diacetilactis*)
Swiss	*Streptococcus salivarius* subsp. *thermophilus*, *Lactobacillus helveticus* or *Lb. delbrueckii* subsp. *bulgaricus* or *Lb. delbrueckii* subsp. *lactis* and *Propionibacterium* spp.
Parmesan	*S. salivarius* subsp. *thermophilus*, *Lb. helveticus* or *Lb. delbrueckii* subsp. *bulgaricus* or *Lb. delbrueckii* subsp. *lactis*
Mozzarella	*S. salivarius* subsp. *thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus* or *Lb. helveticus*
Roquefort	*S. salivarius* subsp. *thermophilus*, *L. lactis* subsp. *lactis* and subsp. *cremoris*, *L. lactis* subsp. *lactis* biovar. *diacetilactis*, *Penicillium roqueforti*
Gouda, Edam	*L. lactis* subsp. *lactis* and subsp. *cremoris* and B or BD flavour cultures*
Cottage cheese	*L. lactis* subsp. *lactis* and subsp. *cremoris*

*B = *Leuconostoc mesenteroides* subsp. *cremoris*/ *Leuconostoc lactis*
D = *Lactococcus lactis* subsp. *lactis* biovar. *diacetilactis*
BD = both Leuconostocs and *L. lactis* subsp. *lactis* biovar. *diacetilactis* are included.

The extracellular proteinase

THE ENZYME

Casein degradation in *L. lactis* is initiated by the extracellular cell envelope-associated serine proteinase, PrtP, which shows considerable homology with the subtilisins of different *Bacillus* species. PrtP is synthesized as an inactive pre-pro-protein. Its activation is catalysed by the maturation protein PrtM. Both the presence of PrtM and the proteolytic activity of PrtP are necessary for the activation of PrtP (Kok and de Vos, 1994). Recently it has been shown that PrtM has some homology to the proline isomerase PpiC of *Escherichia coli*, suggesting that the conformational change of a proline residue could trigger the autoproteolytic activation of PrtP (Rudd *et al.*, 1995).

SPECIFICITY OF PrtP

Biochemical characterization has shown that PrtPs from different strains of *L. lactis*

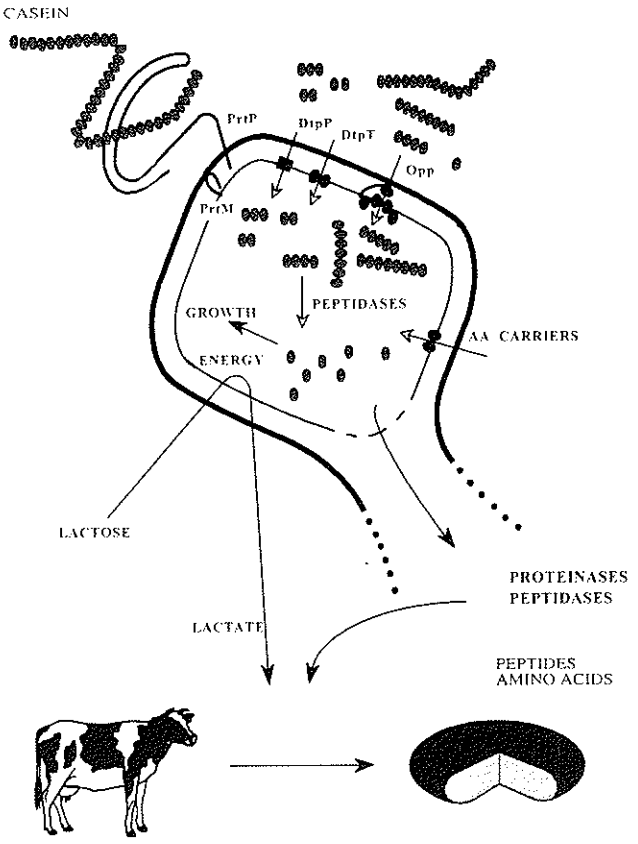

Figure 1. The proteolytic system of *Lactococcus lactis* and its role for growth in milk and for cheese ripening.

differ in their specificity towards casein. Initially, two extremes in substrate specificities have been identified, designated PI and PIII. The PI-type proteinase, represented by the enzymes of the strains HP and Wg2, primarily hydrolyses β-casein and only to a limited extent α_{s1}– and κ-casein. The PIII type, represented by the enzymes of the strains SK11 and AM1, degrades α_{s1}–, κ- and β-casein, but the latter with a different specificity as compared to the PI proteinase (Klein *et al.*, 1995). Recently, the specificity of PrtP of 16 different lactococcal strains was re-examined using the α_{s1}-casein fragment 1–23. Seven groups with specificities varying between the extremes of the PI and the PIII proteinases were identified, indicating an almost free variation of specificities rather than two fixed types (Exterkate *et al.*, 1993). Whether this variation in substrate specificity has a physiological or other function (e.g., in cheese ripening) or is a mere evolutionary play with a not strictly conserved function remains to be elucidated.

A question of prime importance for the understanding of growth of *L. lactis* in milk concerns the size of the peptides resulting from degradation of casein by PrtP. In early investigations with both PI and PIII proteinases it was found that PrtP degrades β-casein into peptides with 3 to 25 amino acid residues (for an overview

see Tan *et al.*, 1993a). Only a few of these peptides were small enough to be translocated by the Opp system, which was thought to transport only peptides with 4 and 5 amino acid residues, according to some authors (Konings *et al.*, 1989), and with a maximum of 6 amino acids according to others (Smid *et al.*, 1991). Therefore, the larger peptides would have to be further degraded by extracellularly located peptidases in order to be suitable substrates for either the di- and tripeptide permease or the Opp system (Smid *et al.*, 1991). Recently, β-casein degradation by a PI-type proteinase was reinvestigated with special emphasis on the small products. Using HPLC coupled to online mass spectrometry, more than 100 different oligopeptides with 4 to 30 amino acid residues were identified. No di- or tripeptides and only traces of free phenylalanine were detected. Eighteen of the oligopeptides were 4–8 residues in size and contained all 20 amino acids (Juillard *et al.*, 1995a). Furthermore, in several genetic and biochemical studies the Opp transport system was shown to be able to translocate oligopeptides with up to at least 8 amino acid residues, and to be essential for *L. lactis* to grow in milk (Kunji *et al.*, 1995; Tynkkynen *et al.*, 1993: for a detailed discussion see below). In contrast to earlier speculations, these data indicate that extracellular casein degradation is catalysed by PrtP alone and that there is no need to postulate extracellular peptidases. A sufficient amount of the oligopeptides can be directly translocated into the cell and further degraded to provide the amino acids needed for growth. These observations are supported by the fact that all of the peptidases characterized so far are located intracellularly (see below).

GENETICS OF *prtP* AND *prtM*

PrtP and PrtM are encoded by the genes *prtP* and *prtM*, respectively. In all cases studied so far, both genes are adjacent to each other and are transcribed divergently. In all except one case, *prtP* and *prtM* are present on plasmids. This observation explains the often reported instability of the proteolytic capacity of lactococcal starter strains. The *prtP* and *prtM* genes of many lactococcal strains have been characterized by restriction enzyme analysis and in a number of cases the genes have been cloned and (partially) sequenced. The results suggest that in lactococci only one major extracellular proteinase is present and that there are only minor differences in the primary structure of proteinases from different strains. For instance, the deduced amino acid sequences of the proteinases of the strains Wg2 and SK11 are 98% identical and those of Wg2 and NCDO763 are 99% identical (Kok and de Vos, 1994; Kok, 1990). The genes and their products have been analysed extensively, which has led to a detailed understanding of their structure and function (for a thorough recent review see Kok and de Vos, 1994).

Differences in PrtP activity in cells obtained from different growth media and the presence of a conserved stem and loop structure in the intergenic promoter region of *prtP* and *prtM* suggested that the activity of these genes may be regulated. Recently, studies with transcriptional gene fusions and quantitative primer extension experiments have shown that the activity of both *prtP* and *prtM* can be repressed in cells grown in whey medium when either peptide mixtures or the dipeptides Pro-Leu or Leu-Pro are added (Marugg *et al.*, 1995, 1996). The role of this regulatory mechanism for growth of lactococci in milk remains to be solved.

Transport of peptides and amino acids

In the acquisition of the essential amino acids, transport of either peptides or free amino acids is crucial. Up to now one transport system for oligopeptides (Opp), two transport systems for di- and tripeptides (DtpT, DtpP) and several systems for the translocation of amino acids have been identified in *L. lactis*.

THE OLIGOPEPTIDE TRANSPORT SYSTEM

In *L. lactis*, oligopeptides are translocated by an ATP-driven transport system which consists of five subunits, i.e., two transmembrane proteins OppB and OppC, two ATP-binding proteins OppD and OppF and a membrane-linked peptide-binding lipo-protein OppA. It has been shown that this system can translocate peptides with four to up to at least eight residues, but no conclusive experiments have been reported which would indicate the upper size limit of this system. The genes of the Opp system are organized in an operon, *oppDFBCApepO*, which has been cloned and sequenced. Two interesting features of this operon are that, first, immediately upstream of *oppA* another promoter may be present indicating that this gene is expressed and/or regulated independently from the first four genes and, second, the last gene of the *opp* operon is an oligopeptide endopeptidase. The possible functional significance of the latter observation remains to be elucidated. To study the function of Opp, both an *oppA* disruption mutant was made and the entire operon has been deleted (Kunji *et al.*, 1996; Tynkkynen *et al.*, 1993: for discussion of the results see below).

DI/TRIPEPTIDE TRANSPORT SYSTEMS

In *L. lactis* two transport systems have been identified which can translocate di- and tripeptides. The proton-motive-force-driven DtpT has a more general specificity with preferences for hydrophilic and charged peptides, whereas DtpP has a preference for hydrophobic peptides, especially those containing branched chain amino acids and is dependent on ATP or another energy-rich phosphorylated compound. The gene encoding DtpT has been cloned and sequenced and a targetted deletion mutant has been constructed. Using the toxic dipeptide Phe-β-chloro-Ala a mutant of DtpP as well as a *dtpTdtpP* double mutant were also constructed (Foucaud *et al.*, 1995; Hagting *et al.*, 1994; for discussion of the functional analysis of these mutants see below).

AMINO ACID TRANSPORT

Nine transport systems for 16 proteinogenic amino acids (except Cys, Asp, His and Met), have been identified by biochemical methods in *L. lactis*. So far only a mutant has been constructed for the Ala transporter by using the toxic β-chloro-alanine (Poolman, 1993). Growth experiments with chemically defined medium supplemented with amino acids showed that the various transport systems are sufficiently active to allow growth of *L. lactis* on amino acids alone. The genetic analysis of the various amino acid transporters is the next logical step to understand more of their function for growth of *L. lactis* in milk and in other media.

ROLE OF PEPTIDE TRANSPORT IN GROWTH OF *L. LACTIS* IN MILK

One important question concerning the proteolytic pathway of *L. lactis* was the actual involvement of the known peptide transport systems in the acquisition of essential amino acids. Several independent studies have shown that Opp plays a crucial role and may be the only transporter needed in this process: (1) PrtP releases from (β-)casein only peptides with four and more residues (Juillard *et al.*, 1995a); (2) no extracellular peptidases have been identified (see below); (3) in transport experiments using casein and PrtP-containing cells, amino acid accumulation is blocked when Opp is inactivated but not when DtpT was eliminated (Kunji *et al.*, 1995); and (4) only inactivation of Opp leads to severe impairment of growth of *L. lactis* in milk with final cell densities one tenth of those reached by the wild-type strain. Inactivation of DtpT has no effect on growth (Kunji *et al.*, 1995). Further genetic and biochemical analyses need to be carried out to reveal the *in vivo* function of the two di/tripeptide transporters for growth in milk. One possible function for DtpT would be peptide excretion when a critical internal concentration of peptides is reached.

The peptidases of *L. lactis*

The third and last step in the proteolytic pathway of *L. lactis* is the degradation to amino acids of peptides which have entered the cell. *Table 2* gives a summary of the lactococcal peptidases which could be involved in peptide degradation and their main characteristics. The peptidases can be subdivided in two major groups, namely (1) endopeptidases, which hydrolyse their substrate endolytically, and (2) aminopeptidases, which need a free N-terminus and cleave off either one or two amino acids from this end of a peptide. No carboxypeptidases, which degrade peptides from their C-termini, have been found in lactococci (Kok and de Vos, 1994).

Whether *L. lactis* has more peptidases than those listed in *Table 1* remains to be investigated. Also, little is known about the variation in this set of enzymes in different lactococcal strains. Another interesting question is whether there is a correlation between the peptidase spectrum of a strain and the specificity of the strain's extracellular proteinase.

PEPTIDASE GENE CLONING

The genes of most of the peptidases listed in *Table 2* have been cloned and their nucleotide sequences have been determined. The nucleotide and derived amino acid sequence information has been used to identify sequence similarities with other genes or proteins, to map the peptidase genes on the lactococcal chromosome, to identify sequences which are involved in transcription and translation of the genes, to construct mutants for functional analyses (see *Table 2*), and to overexpress the genes in homologous and heterologous hosts. Peptidase genes can be monocistronic (e.g., *pepN*) as well as part of operons (e.g., *pepF, pepO, pepT*) (for references see *Table 2*) and they are distributed randomly over the entire chromosome (Le Bourgeois *et al.*, 1995). The genetic analyses of many of the peptidase genes is not yet complete. Transcription units and transcription start sites remain to be established in a number of cases (e.g., for *pepF, pepT, pepV* and *pcp*). Furthermore, only little is known about

possible regulation of peptidase genes. First results indicate that, like *prtP*, *pepN* and *pepX* are repressed in the presence of increasing peptide concentrations (Meijer *et al.*, 1995). Peptidase expression (PepN, PepT, PepC, PepV, and PepX) during exponential growth in milk was measured in mutants in which up to five peptidase genes had been inactivated. No significant differences with the wild-type strain were detected, although many of the mutants grew slower than the wild-type. This indicates that during exponential growth in milk the peptidase genes are not regulated (Mierau *et al.*, 1996).

CELLULAR LOCALIZATION OF PEPTIDASES

Peptidase localization has been studied in several ways. First, analysis of cellular fractions by Western blotting showed that PepN, PepC, PepX, PepO, and PepT are present inside the cell. However, both immunogold labelling and analysis of membrane vesicles indicated that PepX, PepO and PepT are located in the vicinity of the cell membrane (Tan, 1992a). Second, analysis of deduced amino acid sequences of peptidases revealed that none of these contained a signal sequence for secretion or a membrane spanning domain (for references see *Table 2*). N-terminal amino acid sequencing of purified PepF, PepO, PepN, PepC, PepT, PepA, and PepX showed that, indeed, the amino acid sequences corresponded to the start of the respective primary translation products (for references see *Table 2*). This suggests an intracellular location for all of these enzymes. Third, growth experiments and peptide transport studies sensitively monitor extracellular peptidase activity. When a mutant defective in the oligopeptide transport system was grown on peptides with four to eight amino acids, which in *L. lactis* can only be translocated by this system, no growth was observed (Tynkkynen *et al.*, 1993). Therefore, there is no need to postulate extracellular peptidases which would degrade these peptides into di- and tripeptides which would then be taken up by DtpT or DtpP. Intracellular accumulation of amino acids from casein was studied in Prt$^+$ strains in which different transport systems had been inactivated. A strain with an insertion in *oppA* did not accumulate amino acids, whereas in the wild-type strain and in the *dtpT* mutant amino acids accumulated, confirming that no significant peptidase activity is present outside the cell (Kunji *et al.*, 1995).

ANALYSES OF THE FUNCTION OF LACTOCOCCAL PEPTIDASES *IN VIVO*

One way to study the function of peptidases for growth of *L. lactis* is to analyse mutants lacking one or several peptidases. In a first approach, single mutants have been constructed and their growth studied in milk and in defined medium. Mutants lacking PepF, PepO, PepN, PepC, PepT, PepA, or PepX did not show significant differences in growth rates and acid production during growth in milk when compared to the wild-type strain (de Vos and Siezen, 1994; Erra-Pujada *et al.*, 1995; I'Anson *et al.*, 1995; Mayo *et al.*, 1993; Marugg *et al.*, 1996; Mierau *et al.*, 1994; Monnet *et al.*, 1994). A possible exception is the PepA-deficient mutant which was reported to have a prolonged lag phase. These observations indicated that the activity of these peptidases can either be replaced by other peptidases in the cell or that they are not involved in the degradation of casein-derived peptides.

Table 2. Peptidases of *Lactococcus lactis*

Enzyme/Strain*	Enzyme class	MW [kD]*	Native enzyme	Substrate	Localization	Gene	Mutant	Reference
ENDOPEPTIDASES								
PepF/ *L.l.c.* NCDO 763	metallopeptidase	70	monomer	bradykinine	intracellular	cld + sqd [a]	yes [b]	Monnet *et al.*, 1994
PepF2/ *L.l.c.* MG1363	metallopeptidase	70	monomer [c]	bradykinine	intracellular [c]	cld + sqd	– [c]	Nardi *et al.*, 1995
PepO/ *L.l.c.* Wg2, CI3, SK11, MG1363, P8-2-47, SSL135	metallopeptidase	71.5	monomer	metenkephalin	intracellular	cld + sqd P8-2-47	yes MG1363	Baankreis, 1992; Mierau *et al.*, 1993, 1996; Pritchard, 1994; Tan *et al.*, 1991
PepO2/ *L.l.c.* MG1363	metallopeptidase	71.8	monomer [d]	metenkephalin [d]	intracellular [d]	cld + sqd	–	Hellendoorn *et al.*, unpublished results
AMINOPEPTIDASES								
Broad specificity aminopeptidases								
PepN/ *L.l.c.* Wg2, HP, MG1363	metallopeptidase	95	monomer	1⇓2–3–4. . .[5]	intracellular	cld + sqd, MG1363, Wg2	yes, MG1363	de Vos and Siezen, 1994; Exterkate *et al.*, 1992; Mierau *et al.*, 1996; Strøman, 1992; Tan and Konings, 1990; Tan *et al.*, 1992a; van Alen-Boerrigter *et al.*, 1991
PepC/ *L.l.c.* AM2, MG1363	thiol-aminopeptidase	50	hexamer	1⇓2–3–4. . .	intracellular MG1363	cld + sqd, AM2, MG1363	yes,	Chapot-Chartier *et al.*, 1993; Mierau *et al.*, 1996; Neviani *et al.*, 1989
PepT/ *L.l.c.* Wg2, AM2, MG1363	metallopeptidase	46	dimer	1⇓2–3	intracellular	cld + sqd, MG1363	yes, MG1363	Bacon *et al.*, 1993; Bosman *et al.*, 1990; Mierau *et al.*, 1994
Peptidase 53/ *L.l.c.* IMN-C12	thiol-peptidase (	23	trimer	1⇓2–3 1⇓2, 1⇓2–3–4)	cell-wall	–	–	Sahlstrøm *et al.*, 1993
PepV/ *L.l.c.* H61, Wg2 MG1363 *L.l.d.* CNRZ267	metallopeptidase	49	monomer	1⇓2	intracellular	cld + sqd, MG1363,	–	Desmazeaud and Zevaco, 1977; Fayard, unpublished results; Hwang *et al.*, 1981; van Boven *et al.*, 1988

Specific task aminopeptidases

PepA/ *L.l.c.* HP, NCDO712, AM2, MG1363	metallopeptidase	38	trimer hexamer [c]	Glu⇓2–3. . .; Asp⇓ 2–3. . .; Ser⇓2–3. . .	intracellular	cld + sqd, NCDO712	yes, MG1363	Bacon *et al.*, 1994; Exterkate and de Veer, 1987; l'Anson *et al.*, 1995; Niven, 1991
PCP/ *L.l.c.* HP, MG1363	serine-peptidase or thiol-peptidase [g]	25 [f]	–	Pyr-Glu⇓2–3. . .	intracellular	cld + sqd, MG1363	–	Baankreis, 1992; Exterkate, 1977; Haandrikman, unpublished results
PepXP/ *L.l.c.* P8-2-47, NCDO763, AM2, MG1363, *L.l.l.* H1	serine-peptidase	88	dimer	1-Pro⇓3–4. . .	intracellular	cld + sqd, P8-2-47, NCDO763	yes, MG1363	Booth *et al.*, 1990a; Kiefer-Partsch *et al.*,1989; Leenhouts *et al.*, 1996; Lloyd and Pritchard, 1991; Mayo *et al.*, 1991, 1993; Nardi *et al.*, 1991; Zevaco *et al.*, 1990
PepP/ *L.l.c.* NCDO763	metallopeptidase	43	monomer	1⇓Pro-Pro-4. . .	–	–	–	Mars and Monnet, 1995
PIP/ *L.l.c.* HP	metallopeptidase	50	dimer	Pro⇓2(–3)	intracellular	–	–	Baankreis and Exterkate, 1991
PRD/ *L.l.c.* H61, AM2	metallopeptidase	42	monomer	1⇓Pro	intracellular	–	–	Booth *et al.*, 1990b; Kaminogawa *et al.*, 1984

[a] *L.l.l.*: *Lactococcus lactis* subsp. *lactis*; *L.l.d.*: *L. lactis* subsp. *lactis* biovar. *diacetilactis*; *L.l.c.*: *L. lactis* subsp. *cremoris*
[*] If available, this information is based on the derived amino acid sequence of a cloned gene.
[w] Not determined or not done.
[s] The numbers indicate amino acid residues counted beginning from the N-terminus of the peptide.
[d] If not indicated otherwise the gene was cloned (cld) and/or sequenced (sqd) from the same strain from which the peptidase was purified.
[b] If not indicated otherwise the mutant was constructed in the strain from which the gene was cloned.
[c] Inferred from the high sequence similarity to PepF
[d] Inferred from the high sequence similarity to PepO
[e] Conflicting reports.
[f] Determined from the nucleotide sequence of the *pcp* gene.
[g] Homologous PCP from other bacteria are thiol-peptidases (Gonzales and Robert-Bandouy, 1994)

Peptide degradation in *pepN* and *pepX* mutants has been studied using cell-free extracts. The *pepN* mutant was unable to degrade Lys-, Pro-, Phe-, Met- and Arg-*p*-NA, whereas degradation of Glu-, Ala-Pro-, and Pyr-*p*-NA was unaffected. Degradation of peptides with four and more amino acid residues was significantly decreased, whereas di- and tripeptides were degraded normally. Consistently, in minimal medium in which the essential Met was supplied as part of a tri- or tetra-peptide, the growth rate of the *pepN* mutant was lower than when Met was given as free amino acid or as part of a dipeptide (Baankreis, 1992). With a *pepX* mutant, a change in the breakdown pattern of Tyr-Gly-Gly-Phe-Met (Met-enkephalin) was observed (Mayo *et al.*, 1993). These data showed that both PepN and PepX are involved in peptide degradation and that PepN, under certain circumstances, plays a role in the acquisition of essential amino acids from externally supplied peptides.

MULTIPLE PEPTIDASE MUTANTS

To study the function of the various peptidases for growth of *L. lactis* in milk one has to take into account that, first, the cell is supplied with a multitude of peptides (see above) which can serve as alternative amino acid sources and, second, that many peptidases are present in the cell which could, at least to a certain extent, replace each others' activities. Reduction of the number of peptidases and/or the use of a medium less complex than milk would provide experimental conditions needed to allow detailed examination of the role of the different peptidases in cell nutrition. A set of sixteen mutants was constructed in which up to all five of the following peptidase genes had been inactivated: *pepO, pepN, pepC, pepT* and *pepX* (Mierau *et al.*, 1996). The study of these mutants led to a first insight in the function of the peptidolytic system of *L. lactis in vivo*, as will be detailed now.

Growth of the mutants was not affected in either M17 or in chemically defined medium (CDM) with all twenty amino acids, indicating that the mutations *per se* are not deleterious for the cell. In milk, in which growth depends on the degradation of casein-derived peptides, deletion of an increasing number of peptidase genes leads to decreasing growth rates. A strain which lacks all five peptidases grows more than 10 times slower than the wild-type. The main outcome of these experiments is that growth rates decrease gradually when the number of peptidase mutations is increased, whereas the final cell densities reached by the strains stays largely the same (except for the *pepXpepTpepOpepN*, *pepXpepTpepNpepC* and *pepXpepTpepOpepCpepN* mutants which have very slow growth rates). The results indicate that the casein-derived peptides can still be degraded under these circumstances but at a lower rate (Mierau *et al.*, 1996).

In a second set of experiments, growth of the mutants was tested in chemically defined medium (CDM) in which the essential amino acid Leu was supplied as part of a peptide so that growth depends on the capacity of the cell to hydrolyse this peptide. Under these defined conditions certain mutants did not grow at all. By comparing the growth of different mutants on a certain peptide it was possible to identify individual peptidases responsible for the degradation of that particular peptide. The results of these growth experiments showed that only a limited set of peptidases and in some cases only one peptidase is capable of degrading a given peptide. For instance the tripeptide Leu-Gly-Gly is degraded by either PepT or PepN while Ala-Pro-Leu is

mainly degraded by PepX and only to a very limited extent by other peptidases. Furthermore, the tetrapeptide Gly-Leu-Gly-Leu is degraded either by PepN or by a combination of PepC and PepT. If PepN is inactivated together with either of the other two peptidases, the mutant strain can not grow on the tetrapeptide. This experiment shows that apart from these three peptidases no other enzymes are present in the cell which can efficiently hydrolyse Gly-Leu-Gly-Leu (Kunji *et al.*, 1996).

The results from these two types of growth experiments lead to the following model of peptide degradation in *L. lactis* as part of the proteolytic pathway allowing the organism to use extracellular protein (casein) as source of nutritional amino acids (*Figure 2*). In the first step only one enzyme, namely PrtP, breaks down casein into peptides of 4 to 30 amino acid residues (Juillard *et al.*, 1995a). Next, peptides from this pool are translocated into the cell by Opp and form the substrate for the peptidases present in the cell (Juillard *et al.*, 1995b; Kunji *et al.*, 1995; Tynkkynen *et al.*, 1993). Subsequently, the peptides are broken down by the cooperative action of the peptidases into amino acids, which can than be used to generate new cell material. Each peptide is hydrolysed by the set of enzymes which has the highest overall rate towards this peptide and its subsequent breakdown products. The peptides are first broken down by peptidases with broad specificities, both from the N-terminus (PepN and PepC) and endolytically (PepO), leading to smaller peptides as well as to free amino acids. The final steps are most probably performed by the general but size-specific peptidases PepT and PepV. Specific peptides with either Pro in the first or second position, or with Glu, pyro-Glu or Asp in the first position are degraded by specific peptidases (PepX, PIP, PRD, PepP, PepA, and PCP). At least some of the general aminopeptidases have overlapping specificities (PepN, PepC and PepT) so that they can take over each others' functions. Although these enzymes can replace each other, they do not necessarily degrade a peptide with the same overall rate, leading to the observed lower

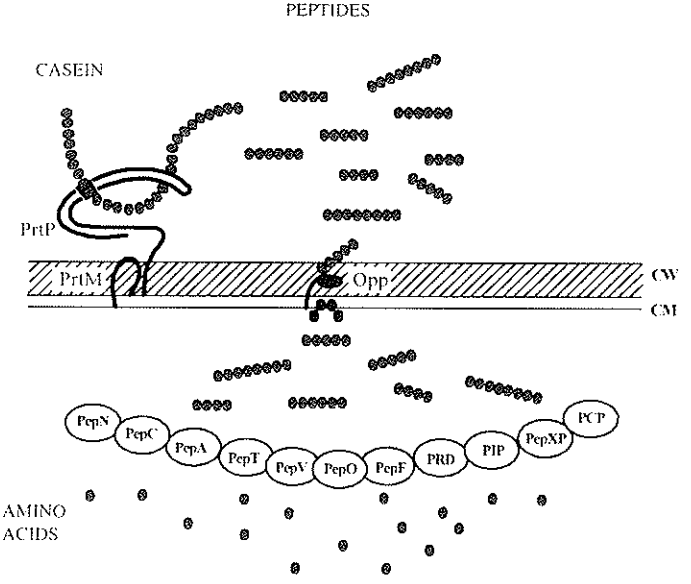

Figure 2. Model of the degradation of casein and casein-derived peptides by *Lactococcus lactis*.

growth rates of mutants in milk (Kunji *et al.*, 1996). It will take more mutants and growth experiments with a larger set of peptides to work out the function of all the different peptidases present in *L.lactis* in detail.

The proteolytic system of other lactic acid bacteria

Fast progress is being made in the investigation of the proteolytic system of various *Lactobacillus* species and of *Streptococcus salivarius* subsp. *thermophilus*. *Tables 3, 4, 5* and *6* give an overview of the enzymes which have been identified so far, and their main characteristics. Interestingly, a few enzymes have been identified which have not been observed in *L. lactis*, such as e.g., PepL in *Lb. delbrueckii* and other types of proteinases in *Lb. delbrueckii* and *Lb. helveticus* (for references see the tables). In most of the cases the peptidases have been localized to the cytoplasm. In accord with this location is the absence of a signal sequence or membrane-spanning domain in any of the available deduced amino acid sequences.

Since *Lactobacilli* are less amenable to genetic manipulation, only in a few cases could the function of the peptidases be studied *in vivo*. Defined mutants in *pepX, pepN* and *pepC* are available in *Lb. helveticus*. Growth experiments in milk showed that *pepX*, *pepX pepC*, or *pepXpepCpepN* mutants had growth rates lower than the wild-type strain, indicating that these peptidases might be important for the release of amino acids from casein-derived peptides (J.L. Steele, personal communication).

Comparison of the data obtained for *L. lactis* and for the different *Lactobacillus* species and *S. salivarius* subsp. *thermophilus* indicate that the proteolytic systems of these bacteria are very similar.

The role of the proteolytic system of *L. lactis* in cheese making

An important process during cheese ripening is proteolysis as it contributes to development of texture, taste and flavour of a cheese. Proteolysis during ripening can be subdivided in two phases: (1) the degradation of casein or its larger subfragments by rennet proteinase(s), indigenous milk proteinases and starter proteinases (e.g., PrtP) into peptides, and (2) the degradation of these peptides in smaller peptides and amino acids. Small peptides and amino acids are either flavour components themselves or serve as precursors in further enzymatic or chemical reactions leading to flavorous compounds (*Figure 1*) (Lawrence *et al.*, 1987; Olson, 1990). Cheeses made with proteinase-negative (Prt$^-$) starters have little or no flavour (Exterkate and Alting, 1995; Law *et al.*, 1993; Stadhouders *et al.*, 1988). The role of the various peptidases of *L. lactis* in cheese ripening is less well understood. In a first set of experiments the role of PepN and PepX in cheese ripening was analysed. A cheese made with a starter containing 90% of a *pepN* mutant had a clear bitter defect, demonstrating the debittering effect of PepN *in vivo* (Baankreis, 1992). Similar observations have been made *in vitro* in which a casein hydrolysate was shown to be debittered by PepN (Tan *et al.*, 1993b). Lack of PepX activity in the starter bacteria led to a decrease of the organoleptic quality of cheese (Baankreis, 1992). Furthermore, casein degradation products detected in experimental cheeses made with proteinase-negative starters pointed to the action of an endopeptidase, which was in one case identified to be PepO (Baankreis, 1992; Exterkate and Alting, 1995).

Table 3. Components of the proteolytic system identified in *Lactobacillus delbrueckii*

Enzyme/Strain*	Enzyme class	MW [kD]*	Native enzyme	Substrate	Localization	Gene	Mutant	Reference
Proteinase/ *L.d.b.*	metalloproteinase	–[g]	–	casein	cell wall	–	–	Stefanitsi *et al.*, 1995
PrtB/ *L.d.b.*, CNRZ397, ACA DC235, NCDO1489	serine-proteinase	170	monomer	α – and β-casein	cell wall	cld + sqd, [a] NCDO1489	yes, NCDO1489	Gilbert, *et al.*, 1996; Laloi *et al.*,1991; Stefanitsi *et al.*, 1995
PepN/ *L.d.l.* DSM7290	metallopeptidase	95	monomer	Lys⇓*p*-NA	intracellular [b]	cld + sqd	–	Klein *et al.*, 1993
PepN/ *L.d.b.*	metallopeptidase	95	monomer	Lys⇓*p*-NA	–	–	–	Bockelman *et al.*, 1992
PepL/ *L.d.l.* DSM7290	serine-peptidase	35	–	Leu⇓*p*-NA	intracellular [c]	cld + sqd	–	Klein *et al.*, 1995
PepC/ *L.d.b.* B14	thiol-aminopeptidase	54	monomer	1⇓2–3[s]	–	–	–	Wohlrab and Bockelman, 1993
PepC/ *L.d.l.* DSM7290	thiol-aminopeptidase	51	–	Leu⇓β-NA	–	cld + sqd	–	Klein *et al.*, 1994b
Dipeptidase/ *L.d.b.* B14	metallopeptidase	51	monomer	1⇓2	–	–	–	Wohlrab and Bockelman, 1992
PepV/*L.d.l.* DSM7290	metallopeptidase	52	–	1⇓2	intracellular [b]	cld + sqd	–	Vongerichten *et al.*, 1994
PepX/ *L.d.b.* CNRZ397, B14	serine-peptidase	95	dimer	Ala-Pro⇓*p*-NA	intracellular	–	yes [d], CNRZ397	Atlan *et al.*, 1990; Bockelman and Fobker, 1991
PepX/ *L.d.l.* DSM7290	serine-peptidase	88	monomer	Ala-Pro⇓*p*-NA	intracellular [b]	cld + sqd	–	Meyer-Barton *et al.*, 1993
PepIP/ *L.d.b.* CNRZ397	serine-peptidase	33	trimer	Pro⇓2(–3)	cell envelope [c]	cld + sqd	–	Atlan *et al.*, 1994; Gilbert *et al.*, 1994
PepI/ *L.d.l.* DSM7290	serine-peptidase	33	–	Pro⇓*p*-NA	intracellular [b]	cld + sqd	–	Klein *et al.*, 1994a
PepQ/ *L.d.l.* DSM7290	metallopeptidase	41	–		1⇓Pro	intracellular [b]	cld + sqd	– Stucky *et al.*, 1995

[#] *L.d.b.*: *Lactobacillus delbrueckii* subsp. *bulgaricus*; *L.d.l.*: *Lactobacillus delbrueckii* subsp. *lactis*.

[*] If available, this information is based on the derived amino acid sequence of a cloned gene.

[g] Not determined or not done.

[s] The numbers indicate amino acid residues counted beginning from the N-terminus of the peptide.

[a] If not indicated otherwise the gene was cloned (cld) and/or sequenced (sqd) from the same strain from which the peptidase was purified.

[b] Inferred from the fact that no signal sequence or membrane spanning domain was found.

[c] Most of the enzyme has been found in the cytoplasm, but also membrane association is considered since a stretch of hydrophobic amino acids was present at the C-terminus of the deduced amino acid sequence.

[d] Nitroso-guanidine mutant.

[e] This is in contradiction with the fact that no signal peptide was found in the PepIP primary translation product.

Table 4. Components of the proteolytic system identified in *Lactobacillus helveticus*

Enzyme/Strain	Enzyme class	MW [kD][*]	Native enzyme	Substrate	Localization	Gene	Mutant	Reference
Proteinase/ CP790	serine-proteinase	45	–[e]	α-and β-casein	cell wall	–	–	Yamamoto *et al.*, 1993
Proteinase/ L89, CNRZ303	serine-proteinase	180	–	α-and β-casein	cell envelope	–	–	Martín-Hernández *et al.*, 1994; Zevaco and Gripon, 1988
PepN/ 53-7, CNRZ32, ITGL1, SBT2171, LHE-511	metallopeptidase	96	monomer	1⇓2–3–4. . .[§]	intracellular [a]	cld + sqd [b], 53-7, CNRZ32	yes [c] CNRZ32, ITGL1 [d]	Blanc *et al.*, 1993; Christensen *et al.*, 1995; Khalid and Marth, 1990a; Miyakava *et al.*, 1990; Sasaki *et al.*, 1996; Steele, personal communication; Varmanen *et al.*, 1994
PepC/ CNRZ32, 53-7	thiol-aminopeptidase	49	–	1⇓2–3–4. . .	intracellular [a]	cld + sqd, 53-7 CNRZ32	yes, CNRZ32	Fernandez *et al.*, 1994; Steele, personal communication; Vesanto *et al.*, 1994
DIP/ SBT2171, 53-7	metallopeptidase	50	monomer	1⇓2	–	cld + sqd, 53-7	–	Palva, personal communication
PepX/ CNRZ32, 53-7	serine-peptidase	88	dimer	1-Pro⇓3–4. . .	intracellular [a]	cld + sqd, 53-7, CNRZ32	yes, CNRZ32	Khalid and Marth, 1990b; Palva, personal communication, Vesanto *et al.*, 1995; Yüksel and Steele, 1995
PepPN/ CNRZ32, 53-7	–	35	–	Pro⇓2(–3)	–	cld + sqd, 53-7 CNRZ32	–	Dudley and Steele, 1994; Palva, personal communication

* If available, this information is based on the derived amino acid sequence of a cloned gene.
[e] Not determined or not done.
§ The numbers indicate amino acid residues counted beginning from the N-terminus of the peptide.
[a] Derived amino acid sequence of the cloned gene does not have a signal sequence or transmembrane domain.
[b] Cloned and sequenced.
[c] Only present as part of a multiple mutant together with mutations in *pepX* and *pepC*.
[d] Obtained by chemical mutagenesis.

Table 5. Components of the proteolytic system identified in other *Lactobacillus* species

Enzyme/Strain*	Enzyme class	MW [kD]*	Native enzyme	Substrate	Localization	Gene	Mutant	Reference
PrtP/ *L.p.p.* NCDO151, HN14	serine-proteinase	181	–[nd]	β-casein	cell-envelope	cld + sqd [a], NCDO151	–	Holck and Naes, 1992; Kojic *et al.*, 1991; Naes and Nissen-Mayer, 1992
Aminopeptidase/ *L.l.*1183	metallopeptidase	78	monomer	1⇓2–3–4..[s]	cell-envelope[b]	–	–	Eggimann and Bachmann, 1980
Aminopeptidase/ *L.c.c.*LLG	metallopeptidase	87	monomer	Lys⇓*p*-NA	–	–	–	Arora and Lee, 1992
Dipeptidase/ *L.s.*L110	metallopeptidase	50	monomer	1⇓2	–	–	–	Montel *et al.*, 1995
PepX/ *L.c.c.*LLG,	serine-peptidase	79	monomer	1-Pro⇓3–4. . .	–	–	–	Habibi-Najafi and Lee, 1994
PepX/ *L.l.*[x]	serine-peptidase	80	dimer	1-Pro⇓3–4. . .	–	–	–	Meyer and Jordi, 1987

* *L.p.p.*: *Lactobacillus paracasei* subsp. *paracasei*, *L.l.*: *Lactobacillus lactis*, *L.c.c.*: *Lactobacillus casei* subsp. *casei*, *L.s.*: *Lactobacillus sake*

* If available, this information is based on the derived amino acid sequenced of a cloned gene.

[nd] Not determined or not done.

[s] The numbers indicate amino acid residues counted beginning from the N-terminus of the peptide.

[x] Strain not specified.

[a] Cloned and sequenced.

[b] Doubtful since no intracellular marker enzymes were used as controls and because very similar enzymes of other lactic acid bacteria are all located intracellularly.

Table 6. Components from the proteolytic system identified in *Streptococcus salivarius* subsp. *thermophilus*

Enzyme/Strain	Enzyme class	MW [kD]*	Native enzyme	Substrate	Localization	Gene	Mutant	Reference
Proteinase/ CNRZ 385, CNRZ703	serine-proteinase	–[a]	–	α- and β-casein	cell wall	–	yes, CNRZ385	Shahbal *et al.*, 1991, 1993
Endopeptidase/ 160	metallopeptidase	39	monomer	glucagon, insulin β-chain	intracellular	–	–	Desmazeaud, 1974
PepN/ NCDO573, ACA-DC114, CNRZ302	metallopeptidase	98/ 92	monomer	1⇓2–3–4. . .[b]	intracellular	–	–	Midwinter and Pritchard, 1994; Rul *et al.*, 1994; Tsakalidou and Kalantzopoulos, 1992
PepC/ CNRZ302	thiol-aminopeptidase	50	hexamer	1⇓2–3–4. . .	intracellular	cld + sqd [c], CNRZ302	–	Chapot-Chartier *et al.*, 1994a
PepX/[x]	serine-peptidase	80	dimer	1-Pro⇓3–4. . .	–	–	–	Meyer and Jordi, 1987

* If available, this information is based on the derived amino acid sequence of a cloned gene.
[a] Not determined or not done.
[b] The numbers indicate amino acid residues counted beginning from the N-terminus of the peptide.
[x] Strain not specified.
[c] Cloned and sequenced.

Conclusions

The research efforts of the last few years have considerably deepened our knowledge of the proteolytic systems of lactic acid bacteria and especially of the role of the various peptidases in the degradation of casein-derived peptides. Yet, there are still a number of challenges, for example to describe the regulation of the proteolytic systems both of the individual components and of the systems as a whole. Furthermore, the now available peptidase genes (see *Tables 2, 3, 4, 5* and *6*) and mutants as for example described in Mierau *et al.* (1996) open the way to a direct analysis of the role of lactic acid bacteria peptidases in cheese ripening and other (milk) fermentations.

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