The proteolytic system of Lactococcus lactis
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Document Version
Publisher's PDF, also known as Version of record

Publication date:
1997

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
SUMMARY

Lactococci are unable to synthesize a large number of amino acids and are dependent on their uptake to fulfil the need for nitrogen. Milk does not contain sufficient amounts of free amino acids to allow growth of the bacteria to high cell densities (Juillard et al., 1995b). Lactococci, therefore, need to use the proteins present in milk as source of amino acids. Caseins are the major protein source in milk and are organized in large complexes, called micelles. An extensive proteolytic system, putatively consisting of about 30 different proteolytic enzymes and transport systems, is required to liberate amino acids from caseins. The components of the proteolytic pathway can be subdivided into three groups on the basis of their function; (i) proteinases that breakdown caseins to peptides, (ii) transport systems that translocate the breakdown products across the cytoplasmic membrane and (iii) peptidases that degrade peptides to amino acids. The biochemical and genetic properties of putative components of the pathway have been studied extensively by a large number of research groups (Chapter 1). The knowledge of the individual proteins, however, is insufficient to explain the role of each of these enzymes in the pathway. Another unsettled question at the start of the project related to the location of the peptidases. It was always assumed that peptides generated by the proteinase are too big to be transported and hence require further degradation by extracellular peptidases. The genetic and biochemical properties of the currently investigated enzymes suggest that most peptidases are located intracellularly, which implies that peptide transport systems must be present that can translocate the products of the proteinase directly. In this thesis, the properties of the identified peptide transport systems are studied in the context of their role in the utilization of caseins.

To study peptide transport in whole cells, a method was developed to monitor changes in intracellular amino acid and peptide pools (Chapter 2). It combines several earlier described techniques and consists of rapid filtration and perchloric acid extraction of the cells; labelling of amino acids and peptides with the fluorophore dansylchloride and separation of the derivatized amino acids and peptides by high performance liquid chromatography. With this method we were able to show that addition of oligopeptides to energized cells led to accumulation of amino acid residues intracellularly. The use of mutants that were deficient in transport of alanine, di- and trialanine (isolated by selection for resistance for toxic β-CI-alanine and β-CI-alanine-containing di- and tripeptides), allowed us to demonstrate the presence of a system that actively transports oligopeptides. With the use of specific inhibitors it was subsequently shown that oligopeptide transport is driven by the hydrolysis of ATP or a related high-energy intermediate. These observations were substantiated by the discovery of an operon coding for an oligopeptide transport system (Opp) (Tynkkynen et al., 1993). Sequence comparisons revealed that Opp belongs
to the family of ABC-transporters (Higgins, 1992) and consists of a peptide binding protein, two integral membrane proteins and two ATP-binding proteins.

To study the role of peptide transport systems in the utilization of caseins, well-defined mutants were constructed by inactivating the genes for the di-tripeptide transporter (DtpT) and/or Opp (Tynkkynen et al., 1993; Hagting et al., 1994; Chapter 3). Mutants which lacked a functional Opp system, but contained an active proteinase, were unable to grow in milk (Tynkkynen et al., 1993), while growth of mutants lacking DtpT was unaffected in this medium (Kunji et al., 1995; Chapter 3). This observation indicates that one or more essential amino acids enter the cell via uptake through Opp. To substantiate this finding, Lactococcus lactis cells were incubated with the milk protein β-casein and the intracellular pools of amino acids were analyzed. The wild type strain and the DtpT mutant accumulated all amino acids present in β-casein, whereas amino acids were not significantly accumulated by the cells of Opp- and DtpT OPP mutants. When a mixture of amino acids, mimicking the composition of β-casein, was offered to these cells, the amino acids accumulated to a similar extent in all strains. These and other experiments have revealed a number of 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 is transported exclusively by the oligopeptide transport system. 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. 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-derived peptides to amino acids. 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. Growth experiments subsequently showed that uptake of His and Leu-containing peptides by Opp is insufficient to allow growth of L. lactis on this protein substrate.

To study the relationship between peptide transport and degradation, a set of peptidase mutants of L. lactis (Mierau et al., 1996) was used to follow the fate of peptides by chromatographic analysis of intracellular fractions (Chapter 4). Mutants lacking the general aminopeptidases PepN and PepC, the tripeptidase PepT plus the X-prolyl dipeptidyl aminopeptidase PepX could not utilize peptides such as Leu-Gly-Gly, Gly-Phe-Leu, Leu-Gly-Pro, Ala-Pro-Leu and Gly-Leu-Gly-Leu, and these peptides were found to accumulate intracellularly. The observation that peptide transport mutants are impaired in their ability to accumulate peptides (Tynkkynen et al., 1993; Chapter 3), combined with the finding that peptides are translocated into
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The cell as whole entities, proves that peptide transport precedes degradation.

It is clear that both the proteinase and the oligopeptide transport system (Chapter 3) are essential for the utilization of caseins. Although the product formation of the purified proteinases has been studied extensively (Chapter 1), it was unknown what the properties and specificity of cell wall attached proteinase were and whether it would generate a similar peptide pool. Furthermore, no data were available on the natural substrates of the oligopeptide transport system. To study the product formation of the cell-wall attached proteinase without interference of peptidase activity and transport, mutants were constructed that contained PrtP, but lacked the Opp system and the autolysin (to prevent lysis and subsequent release of peptidases; Buist et al., 1995) (Chapter 5). Liquid chromatography coupled to mass spectrometry was used to separate and identify the PrtP-generated peptides. All major products formed by PrtP originated from the C-terminal end of β-casein and were present at the earliest times of degradation. Hydrolytic products derived from the N-terminal end were not detected, while all other parts of β-casein were degraded with low rates. To identify the substrates of the oligopeptide transport system, mutants were constructed that contained Opp but lacked the proteinase. The Opp-dependent disappearance of peptides from the medium and the intracellular accumulation of amino acids and peptides was studied in the wild type and a mutant lacking PepN, PepC, PepT, PepX and the endopeptidase PepO. Specific peptides up to a length of 10 residues disappeared from the medium, while the same peptides or their hydrolytic products were accumulating inside the cells of the five-fold peptidase deficient mutant. Most of the peptides are in the range of 5 to 8 residues, but transport of at least one nonamer and one decamer has been observed. Transport of these peptides into the cell largely explains the intracellular amino acid accumulation observed upon addition of β-casein to wild type cells and supplies the cell with all essential and growth stimulating amino acids with the exception of His. Overall, these studies allowed us to reconstruct the pathway involved in the utilization of β-casein by L. lactis.