Production and utilization of peptides in Lactococcus lactis
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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2002

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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CHAPTER VI

SUMMARY

The inability of lactic acid bacteria to synthesize a large number of amino acids necessitates the presence of a proteolytic system in those environments where protein is the main nitrogen source. For growth in milk, lactococci need the proteolytic system to liberate amino acids from milk proteins (caseins) for optimal growth. The components of the proteolytic system are located in three compartments: an extracellularly located proteinase, three membrane-bound peptide transporters, and a large number of intracellularly located peptidases. The extensive biochemical and genetic studies of the components of this proteolytic system have led to a good understanding of the roles of each of these enzymes in the proteolytic pathway (Chapter 1). In this thesis, we focus on the knowledge accumulated on the production and utilization of peptides in L. lactis.

The sequence of events in the utilization of the milk protein β-casein includes its degradation to oligopeptides by an extracellular proteinase (PrtP), the uptake of the peptides from the casein-hydrolysate by an oligopeptide transporter (Opp), and the degradation of the intracellularly accumulated peptides by a multitude of peptidases. In vitro studies on the utilization of milk proteins with purified proteinase have indicated that, under optimal conditions and extensive incubation, the enzyme is capable of hydrolyzing β-casein into more than 100 different oligopeptides, ranging from 4-up to at least 30 amino acid residues (Juillard et al., 1995). However, it was unknown whether the properties and specificity of cell wall-attached proteinase are the same as those of the purified enzyme. Furthermore, no data were available on the natural substrates of the oligopeptide transport system. To study the product formation of the cell envelope-attached proteinase without interference of peptidase activity and transport, mutants were constructed that contained PrtP, but lacked the Opp system and autolysin (to prevent lysis and subsequent release of peptidases). Liquid chromatography coupled to mass spectrometry (HPLC-MS) was used to separate and identify the PrtP-generated peptides (Chapter 2). The results have revealed that the major PrtP-generated peptides are released from the C-terminal end of β-casein and are formed at the onset of the degradation. The N-terminal end of β-casein remained untouched while other parts of β-casein were degraded at low rates. To identify the substrates of the oligopeptide transport system, the PrtP-generated peptide pool was offered to mutants lacking the proteinase, but containing Opp. The Opp-dependent disappearance of peptides from the medium, and the intracellular accumulation of amino acids and peptides, were studied in the wild type and a mutant lacking PepN, PepC, PepT, PepX and PepO. In general, only a fraction of the PrtP-generated peptides up to a length of 10 residues disappeared from the medium, while the same peptides or their hydrolytic products accumulated inside the cells of the five-fold peptidase deficient mutant. Sequence analysis of those peptides transported via Opp, and the detection of amino acids increasing intracellularly, indicated that all essential and growth stimulating amino acids with the exception of histidine were supplied by the β-casein-derived peptides. These observations are consistent with the inability of the lactococci to acquire histidine from β-casein (Kunji et al., 1995).

The di- and tripeptide transport protein DtpT from L. lactis is unique among bacterial peptide transporters, as it is encoded by a single gene and uses the proton motive force to
drive the transport of di- and tripeptides. Due to the homology of DtpT with important human peptide transporters, *L. lactis* DtpT has received a lot of attention as a model system for the study of structure-function relationship of this class of transporters. The first step towards characterization of DtpT was the solubilization and purification to homogeneity of the histidine-tagged DtpT by pre-extraction of crude membrane vesicles with octaethylene glycol monodecyl ether (C<sub>10</sub>E<sub>8</sub>), followed by solubilization with *n*-dodecyl-β-D-maltoside (DDM) and chromatography on a Ni-NTA resin. The DtpT protein was then functionally incorporated into detergent-destabilized preformed liposomes prepared from *E. coli* phospholipid/Egg phosphatidylcholine. The purification-reconstitution was optimised by testing different detergents to insert DtpT into preformed liposomes, and by controlling the losses of lipids and detergent in the reconstitution process (Chapter 3). Such optimisation of the reconstitution method resulted in proteoliposomes with high and stable proton-symport activities. Under optimised *in vitro* conditions, the DtpT symporter shows high proton motive force-driven transport of di-tripeptides. Moreover, the orientation of DtpT in the membrane could be manipulated by selecting the appropriate detergent to mediate the reconstitution, i.e., the orientation of the DtpT protein in the membrane was random when DDM was used and inside-in with TX<sub>100</sub>.

The uptake of peptides is a well-documented biological phenomenon, which is accomplished by specific, energy-dependent transporters in organisms as distinct as bacteria and humans. Because of its potential as an important drug delivery system, the substrate specificities of mammalian intestinal and renal peptide transport proteins (PepT<sub>1</sub> and PepT<sub>2</sub>) have been studied extensively (Daniel *et al.*., 1992; Döring *et al.*, 1998a, 1998b). However, these studies were carried out under *in vivo* conditions which do not allow easy variation of the transport parameters and the experiments are subject to influences of other transport systems and/or enzymes hydrolysing peptides. The kinetics and substrate specificity of DtpT in proteoliposome was determined using Pro-[<sup>14</sup>C]Ala as a reporter peptide in the presence of various peptides or peptide mimetics (Chapter 4). The results showed that the DtpT protein is specific for di- and tripeptide with the highest affinities for peptides with at least one hydrophobic residue. Free amino acids, ω-amino fatty acid compounds or peptides with more than 3 amino acid residues are not accepted as substrates. Peptides with different side-chains at the 1st, 2nd, or 3rd position have different affinities. Overall, for high affinity interaction with DtpT, the peptides need to have a free amino- and carboxyl-terminus, amino acids in the L-configuration, and trans-peptide bonds. Comparison of the specificity of DtpT with eukaryotic homologues PepT<sub>1</sub> and PepT<sub>2</sub> shows that the bacterial transporter is more restrictive in its substrate recognition. Comparison of the specificity of DtpT with other bacterial homologues indicates that the DtpT of *L. lactis* has a specificity which is equivalent to that of Dpp from *E. coli* and *S. typhimurium*.

It should be stressed that specificity data are obtained from measurements in which the peptides or peptide analogues compete for uptake with Pro-[<sup>14</sup>C]Ala. Inhibition of Pro-[<sup>14</sup>C]Ala uptake does not necessarily imply that the tested substrate is indeed transported by DtpT. In addition, the roles of the di- and tripeptide transport protein DtpT in the proteolytic system is still not well addressed. To deal with this issue under *in vivo* and *in vitro* condition, well-defined mutants have been used in the *in vivo* transport assay of natural degradation products of β-casein by PrtP. Under *in vitro* condition, DtpT reconstituted proteoliposomes were used for the kinetic and substrate specificity studies. The results indicate that the di- and tripeptide transport protein DtpT could play a role for the excretion of di- and tripeptides for the multiple peptidase mutants. Further studies with DtpT proteoliposomes have
confirmed that the DtpT system is able to catalyze the uptake and efflux of a wide range of peptides. A transport cycle includes binding of the substrate at one side of the membrane, translocation of the substrate across the membrane, and release of the substrate at the other side of the membrane. Direct measurements of transport of iodinated tyrosine-containing peptides have shown that the IC50 values determined in the competition assays are comparable to the apparent affinity constants determined for the transport process. These observations indicate that the IC50 values reflect accurately the affinities of DtpT for different di- and tripeptides. Kinetic and substrate specificity studies, using different transport modes, have indicated that PMF-driven uptake and exchange (counterflow) can be used equally well for the kinetic analysis of peptide transport via DtpT (Chapter 5).