Microbial production of thioether-stabilized peptides
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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2010

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Translocation of a thioether-bridged azurin peptide fragment via the Sec pathway in \textit{Lactococcus lactis}

\textbf{Abstract}

This study demonstrates for the first time that a thioether-containing peptide, an azurin fragment, can be translocated via the Sec pathway. This methyl-lanthionine was introduced by the nisin modification enzymes. The Sec pathway can therefore be a successful alternative for those cyclized peptides that are inefficiently transported via NisT.

Azurin, a cupredoxin produced by *Pseudomonas aeruginosa*, can selectively enter human cancer cells and induce apoptosis (229) via binding to the tumor-suppressor protein p53 (3). The azurin peptide fragment p28, containing the amino acids 50-77 (LSTAADMQGVTGDGMASGLDYLKPDD), still enters human cancer cells and inhibits tumor proliferation (197). Importantly, novel cancer treatments can be based on azurin peptide fragments and derivatives thereof (31). Although the pharmacokinetic property of therapeutic peptides is promising, lack of biostability is the major hurdle for their successful application. Consequently, it is very relevant to explore the possibilities for enhancing biostability of these peptides.

In our group we developed a technology to improve the stability of therapeutic peptides by exploiting the nisin synthetase enzymes NisB and NisC for the introduction of thioether bridges. We applied a two plasmid expression system (77, 78, 87), in which the NisBTC-encoding plasmid is compatible with the substrate-peptide-encoding plasmid. *Lactococcus lactis* containing this expression system can secrete non-lantibiotic peptides which are dehydrated or stabilized by a thioether ring (78, 160). NisB dehydrates serines and threonines in substrate peptides, NisC couples dehydrated residues stereo- and regioselectively to cysteines and NisT, the ABC transporter, translocates the modified peptides out of the cell (83, 86, 93, 153). The leader peptide is essential in targeting and modification of the prepeptides (226).

When transport via NisT is impaired or is less efficient, the Sec pathway of *L. lactis* is a successful alternative in translocation of dehydrated peptides. When the nisin leader is preceded by a Sec signal peptide or a Tat signal peptide of respectively 27 or 44 amino acids long, modification by NisB and NisC still occurs (87, 130). However, NisC-cyclized prenisin was not translocated via the Sec system (87). This is likely due to the dimensions of fully modified nisin (14) which is too large to fit in the SecY pore (87, 201). Here, we report for the first time that the Sec pathway of *L. lactis* can translocate a p28 azurin fragment analog with a thioether ring.

We previously demonstrated that under culturing conditions the highly reactive dehydroalanines can spontaneously couple to cysteines, either intra- or extracellularly, whereas the less reactive dehydrobutyrines do not (160). To exclude spontaneous thioether ring formation by dehydroalanines we mutated serines in positions 51 and 67 to alanines, whereas a single dehydratable threonine was kept in position 52 of the 50-77 azurin fragment. Position 56 was mutated to a cysteine to allow posttranslational introduction of a thioether bridge (Table 1, pNZ8048-derived plasmids).
When the azurin peptide fragment fused behind the nisin leader was co-expressed with the enzymes NisB, NisC and NisT in \textit{L. lactis}, no secreted (un)modified peptides in the supernatant were detected. Hence, we made use of the Sec pathway of \textit{L. lactis} for export of the azurin peptide fragment. The nisin leader of the substrate peptide was preceded by the Sec signal peptide of Usp45, SP\textsubscript{Usp45}. This fusion peptide was co-expressed with NisB and NisC in \textit{L. lactis} in the absence of NisT. The used strains and plasmids are listed in Table 1. The culture \textit{L. lactis} NZ9000 (pNG41azurin)(pILBC) was grown in minimal medium. Peptides from 4 ml induced cultures were isolated and purified with bond elute C\textsubscript{18} cartridges from Varian. Dissolved peptides were analyzed by mass spectrometry directly or after TCEP (Tris(2-carboxyethyl)phosphine) - and subsequent CDAP (1-cyano-4-dimethylamino-pyridinium tetrafluoroborate) incubation (160). Mass spectra were recorded with a Voyager DE PRO MALDI-TOF mass spectrometer in the linear mode.

We were able to detect Sec-secreted azurin peptides in the supernatant by mass spectrometry. Some of the dehydrated peptides in the supernatant contained disulfide bonded cysteine adducts meaning that not all the formed dehydrobutyrines were coupled to the peptide’s cysteines (data not shown). This observation of partial ring formation was confirmed by mass spectrometry. Peptides were first reduced with the phosphine TCEP, then alkylated with CDAP; formation of the thiocyanate results in a mass shift of +25 Da. (Fig. 1A). These data indicate that transport of an azurin peptide fragment with a thioether ring via the Sec pathway is possible, when preceded by the SP\textsubscript{Usp45} and the nisin leader.
Fig. 1ABC. Transport of cyclized and dehydrated azurin peptide fragments via the Sec pathway. Culture supernatant was analyzed by Maldi-TOF. Expected mass of the processed, protonated and fully dehydrated fusion peptide (STKDFNLDLVSVSKDSGASPR::LATAADCGVADGMAAGLDKDYLPD) is 5141 Da. (A) Supernatant of *L. lactis* NZ9000 (pILBC)(pNG41azu), fusion peptide preceded by the SP₆₄₄₅. (B) Supernatant of *L. lactis* NZ9000 (pILBC)(pNG51azu), fusion peptide preceded by the SP₈₃₆₇. (C) Supernatant of control *L. lactis* NZ9000 (pILB)(pNG51azu).
As demonstrated before, replacement of the SP\textsubscript{Usp45} with a Tat signal peptide of YwbN from \textit{Bacillus subtilis}, SP\textsubscript{YwbN}, resulted in reduced transport efficiency and simultaneously enhanced extent of dehydration of the substrate peptide (87). To examine the effect of reduced transport on the extent of NisC-mediated cyclization, SP\textsubscript{YwbN} was fused N-terminally to the nisin leader and azurin peptide fragment (Table 1) and coexpressed with NisB and NisC. Peptides in the supernatant of induced cultures were analyzed by mass spectrometry as described above and analyzed on a tricine gel (20) by silver staining (Invitrogen). As a control the substrate peptide was also coexpressed with only NisB. In the case of coexpression of NisB and NisC the secreted peptides were almost fully dehydrated and no cysteinylation were seen (data not shown), suggesting that the peptides were fully ring-closed. TCEP treatment and CDAP incubation (Fig. 1B) of the purified isolated peptides confirmed this observation. As expected, in the supernatant of the control which had only coexpression of NisB (pILB), fully dehydrated peptides were seen. No cyclization took place and therefore all the free cysteines reacted with CDAP (Fig. 1C). This control experiment clearly demonstrated that no spontaneous cyclization had occurred in the absence of NisC. Hence, the thioether bridge formation observed in the experiments presented in Fig 1A and 1B should result from intracellular NisC-mediated cyclization. The data therefore convincingly prove that indeed this intrinsically stable thioether-bridged peptide is transported via the Sec system.

Analyses on silver-stained gel showed that no transport at all via the transporter NisT had occurred when the azurin peptide fragment was preceded by only the nisin leader (Fig. 2, lane 1). In full contrast, transport of the modified azurin peptide fragment via the Sec pathway was successful. The transport of the peptide fragment was less efficient when the SP\textsubscript{YwbN} was used instead of the SP\textsubscript{Usp45} (Fig. 2), which is in full agreement with previous data (87).

![Figure 2. Amount of secreted peptides in the supernatant. Peptides from 2 ml supernatant of induced cultures were applied on a gel. Lanes: M, kaleidoscopic marker (Biorad); 1, NZ9000 (pIL3BTC)(pNZazu); 2, NZ9000 (pIL3BC)(pNG41azu); 3, NZ9000 (pIL3BC)(pNG51azu).](image)

These data demonstrate for the first time that a peptide with an intramolecular thioether bridge can be translocated \textit{in vivo} via the Sec pathway of \textit{L. lactis}. A more in detail studied Sec system is that of \textit{Escherichia coli}. The Sec translocase in the membrane is composed of a highly conserved protein conducting channel, SecYEG (43). The Sec translocase transports unfolded proteins which is driven by the ATPase...
SecA. Homologues of SecYEG and SecA are also found in *L. lactis* (12, 82). *In vitro* studies demonstrated that the translocon SecYEG of *E. coli* can also translocate the polypeptide proOmpA with a disulfide-bridge, which can have a loop of 18 amino acids or smaller (206). Other *in vitro* data demonstrated that proOmpA labeled with bulky fluorescent probes, assessing up to 16 Å were also transported (33). These *in vitro* data with the Sec system of *E. coli* suggested that the SecY translocon is not that rigid and could be used for other purposes such as the *in vivo* translocation in *L. lactis* of peptides with thioether bridges. Interestingly, the efficiency of translocation in *E. coli* can be drastically enhanced by *prlA (secY)* mutations (43). Likely, in the near future such mutations in the SecY translocon of *L. lactis* can contribute to an even more successful application of the Sec pathway for transport of therapeutic peptides with thioether bridges.

_Footnote_
This project was cofinanced by the European Fund for Regional Development and the Dutch Ministry of Economic Affairs.