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Chapter 3

Cloning and Controlled Overexpression of the Gene Encoding the 35 kDa Soluble Lytic Transglycosylase from *Escherichia coli*

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Summary

The lytic transglycosylases of \textit{Escherichia coli} are involved in peptidoglycan metabolism. They resemble lysozymes not only in substrate specificity, but also structurally, as exemplified by the 70 kDa soluble lytic transglycosylase (Slt70). Here we report the cloning of the gene that encodes the 35 kDa soluble lytic transglycosylase (Slt35) of \textit{E. coli}. Based on the sequence of the full-length gene, Slt35 appears to be a proteolytically truncated form of a slightly larger protein. The sequence displays the presence of a consensus lipoprotein signal sequence, and therefore the protein most likely is localized in the outer membrane. Using the T-7 promoter system, Slt35 was overproduced in large quantities and purified to homogeneity for crystallographic purposes. The specific production of 1,6-anhydromuropeptides by the purified protein could be demonstrated.

Introduction

The peptidoglycan layer of bacteria is a polymeric macromolecule that is composed of glycan strands of alternating \textit{N}-acetylglucosamine and \textit{N}-acetylmuramic acid residues, crosslinked by short peptide bridges (for a recent review see (15)). This macromolecule encloses the bacterial cell, thereby serving as a casing that protects the cell from mechanical stress and allows it to maintain a high internal osmotic pressure. Although the protective nature of the peptidoglycan layer implies rigidity, flexibility is required for the cell to be able to grow and divide. These paradoxical characteristics are reflected in a complex metabolic machinery that involves the well-balanced action of a large number of synthesizing and hydrolyzing activities. Of the different enzymes involved, most attention has been given over the years to the penicillin-binding proteins, as it is this class of enzymes that is the direct target of \textit{ß}-lactam antibiotics. The emergence of resistance against this extensively used class of antibiotics has, however, resulted in a quest for new antibacterial targets. The non-penicillin sensitive enzymes involved in the peptidoglycan metabolism can be considered potential candidates. Among these are the lytic transglycosylases, a class of peptidoglycan hydrolyzing enzymes that was originally identified in \textit{E. coli}. Based on the presence of genes homologous to the \textit{E. coli slt} gene in \textit{Enterobacter cloacae} and \textit{Salmonella typhimurium} (2), and the release of the specific products of these enzymes by \textit{Bordetella pertussis} (6) and \textit{Neisseria gonorrhoeae} (19), members of this class are expected to be present in a broad range of gram-negative bacteria. The lytic transglycosylases cleave the \textit{ß}-glycosidic bond between the \textit{N}-acetylglucosamine and \textit{N}-acetylmuramic acid residue, an activity resembling that of the lysozymes, but in addition these enzymes introduce an internal 1,6-anhydro bond in the muramic acid residue of the muropeptide product (13). Of the \textit{E.coli} transglycosylases, the 70 kDa soluble protein (Slt70) has been the most extensively studied, and the three-dimensional structure of this enzyme has recently become available (22). The activity of Slt70, however, does not seem to be essential for
growth of *E. coli* under laboratory conditions as a strain carrying a deletion in the *slt* gene is viable and the only known inhibitor of this enzyme, bulgecin, is not an antibiotic in its own right but only shows synergistic antibacterial activity in combination with β-lactam compounds (21). The reason for this may be the presence of at least two more transglycosylases in *E. coli* that are not inhibited by bulgecin, and may substitute for the activity of Slt70 (12, 16, 23). From an antibacterial point of view, the main objective will therefore be to tackle this class of enzymes as a whole, and for this purpose more functional and structural information is needed about the other members of this enzyme family.

This report describes the cloning of the gene coding for one of those, the 35 kDa soluble lytic transglycosylase (Slt35). After confirmation of the activity of the gene product, a stable form of the protein was purified for further crystallographic studies.

**Materials and methods**

* Molecular cloning methods

General DNA manipulations and cloning procedures were performed according to described protocols (17). Plasmid DNA was prepared using Qiagen (Hilden, Germany) purification systems. Dideoxy sequencing was performed using the Sequenase version 2.0 kit (Amersham, Buckinghamshire, UK), following the manufacturers instructions. The Chameleon system (Stratagene, La Jolla, CA, USA) was used to introduce site-directed mutations. PCR reactions were performed with AmpliTaq DNA-polymerase and reagents from Perkin Elmer (Norwalk, CT, USA) with the following cycling protocol: melting at 94°C for 1 minute, annealing at 45°C for 1 minute, and extension at 72°C for 1 minute for a total of 30 cycles.

* Cloning and sequencing of the Slt35 encoding gene and surrounding region

Two degenerate oligonucleotides (A-11: 5'-CAYAAYGTNATGCARATGGG-3', A12: 5'-RTCRATRAAYTGYTGNGC-3'), the sequences of which were based on the N-terminal amino acid sequence of Slt35 (12), were used to amplify a 63 base pair fragment. The amplified fragment was sequenced, reamplified to incorporate [α32P]-dATP, and was used as a probe in a Southern hybridization procedure on *KpnI* and *PstI* digested genomic DNA of *E. coli* strain 122-1, which has a deletion for the gene coding for Slt70 (15). Two subgenomic libraries were constructed by cloning of genomic DNA fragments of the size range corresponding to that of the hybridization signal into pUC18 (Pharmacia, Uppsala, Sweden). After transformation into super competent XL1-blue (Stratagene, La Jolla, CA, USA), the transformant colonies were screened using the same 63-mer as a probe. One positive clone from each library was selected for further analysis, and the gene coding for Slt35 plus the surrounding region was sequenced by primer walking.

* Overproduction and purification

Using site-directed mutagenesis, an *NcoI* site was constructed, changing the codon for Leu40 into an ATG start codon (primer A62: 5'-CGGCTCAACCATAAGCGCGCCAGACCGG-3'). For selection of the mutant strand, an oligonucleotide
mutating the unique BsAI site in the ampicillin resistance gene of pUC18 was used (primer A44: 5'-GGTGAGCGTGTTCTCGCGGTAT-3'). The 1.9 kb NcoI fragment of the resulting plasmid pAD115 was cloned into the NcoI site of the vector pET21d+ (Novagen, Madison, WI, USA), thereby bringing the expression of Slt35 under the control of the T7 promoter (20). The expression construct pAD121 was transformed into host BL21(DE3) and expression was induced by the addition of 1 mM IPTG. For the large scale purification, 60 grams of induced cells (wet weight) were resuspended in 10 mM Tris-maleate-NaOH buffer pH 7.3, disrupted by passage through an Aminco French pressure cell at 15,000 psi, and the soluble fraction was obtained by centrifugation at 100,000 x g for one hour. The soluble fraction was loaded on a 80-ml SP-sepharose fast flow column (Pharmacia, Uppsala, Sweden) and bound protein was eluted by applying a linear gradient of 0-1 M NaCl in 10 mM Tris-maleate-NaOH. Fractions containing Slt35 (eluting at 0.2 M NaCl) were pooled, dialyzed against 10 mM phosphate buffer pH 6.8, and loaded on a 20 ml Bioscale hydroxylapatite column (Bio-Rad Laboratories, Hercules, CA, USA). Using a linear gradient from 10 mM to 500 mM phosphate, bound proteins were resolved and only the peak fractions containing Slt35 in its purest form (eluting at 150 mM phosphate) were pooled, yielding a total of 310 mg of Slt35.

Site-directed mutagenesis

The codon for Glu206 was exchanged for one coding for a glutamine with primer A75: 5'-CTGTTGATGGCGGGACCAGGACGATCC-3', using the Chameleon system (Stratagene, La Jolla, CA, USA). As this primer also introduced a silent mutation that resulted in the disappearance of a BssHII restriction site, the resulting clones were screened for the absence of this site.

Peptidoglycan hydrolase assay, product analysis and zymography

Peptidoglycan hydrolase activity was determined by measuring solubilization of radiolabeled peptidoglycan polymer following a published procedure (11). HPLC analysis of the produced muropeptides was performed essentially as described (12): isolated peptidoglycan was degraded with purified Slt35 and the released muropeptides were separated by HPLC, using a linear gradient from 0.05% trifluoroacetic acid to 0.035% trifluoroacetic acid containing 20% acetonitrile over 60 minutes. The retention times of the released muropeptides were compared with those of purified monomeric 1,6-anhydromuropeptides, of which the structure had been confirmed by tandem electron-spray mass spectroscopy. Zymogram analysis of crude extracts and purified protein was carried out essentially as described (4), using 10% PAA gels, loaded with 0.03 % purified E.coli peptidoglycan.

Results and discussion

Based on the N-terminal amino acid sequence that had been described (12), a small fragment of the gene encoding the 35 kDa soluble lytic transglycosylase could be amplified. Using this fragment as a probe, two overlapping clones carrying the full-length gene were isolated. Sequence comparison revealed that the first 276 base pairs of the coding region were present in the GenBank database, as being part of the
upstream region of the gut operon, although the authors did not identify the open reading frame (27). This information allowed mapping of the gene at 60.8 minutes on the E. coli chromosomal map, between the gut operon and the recA gene (18) (Fig. 1). As the gene coding for Slt35 is called mltB by other authors that are investigating this protein, we will also adopt this gene name to avoid further confusion in the future.

![FIG. 1. Mapping position of the Slt35-encoding mltB gene. The 60.8 minutes region of the E. coli chromosomal map between alaS and gutA is depicted. The position and direction of transcription of the genes in this region is indicated by the open arrows, whereas the solid arrow represents the 4.8 kb KpnI fragment that was cloned and from which the sequence of the mltB gene was determined.](image)

The primary sequence of the gene product is shown in Fig. 2. Analysis of this sequence revealed that the gene product is very positively charged (a calculated pI of 9.4 for the precursor protein). This positive charge may ensure an optimal interaction with the peptidoglycan substrate, which is highly negatively charged.

The spacing between the published N-terminal sequence and the first possible ATG start codon upstream proved to be unusually large. Although this possible start codon is followed by a stretch of sequence displaying the characteristics of a consensus signal sequence, i.e. a short stretch of sequence containing positively charged residues followed by a longer hydrophobic stretch (24), no consensus signal peptidase cleavage site at the start of the published N-terminal sequence could be detected. Rather, directly following the hydrophobic stretch, a consensus cleavage site for the lipoprotein signal peptidase II is present (25). The cysteine of the consensus cleavage site is separated from the first residue of the published N-terminal sequence by 20 amino acid residues. It therefore seems likely that the protein encoded by the mltB gene is a lipoprotein and that the lipid anchor and the active domain are linked by a proteolysis-prone spacer. Although the lipoprotein character of Slt35 has recently been confirmed (10), the issue of whether the clipping of the linker region is a purification artifact or has physiological relevance, remains to be investigated.

Downstream of the mltB gene a small open reading frame of unknown function was identified and database searching using the BLAST algorithm (1) showed that this open reading frame is significantly homologous to an open reading frame, ydef, downstream of the marB locus (5). (data not shown). This homology sheds no light on the possible function of the protein encoded by the small open reading frame, as the function of ydef is also elusive.
Although the described homology between the N-terminal sequence of Slt35 and a stretch of sequence in Slt70 suggested a high degree of sequence conservation between these proteins (12), the overall homology between the C-terminal part of Slt70 and Slt35 turns out to be less convincing. The sequence of Slt35 does neither show significant homology to the proposed transglycosylase fingerprint (14). As the catalytic mechanism of Slt70 and related proteins involves an essential glutamic acid residue, we attempted to identify possible candidates for this residue in Slt35. It appeared that the catalytic glutamic acid of Slt70 could be aligned with Glu206 of Slt35, resulting in a reasonably good alignment in the region comprising two of the three conserved boxes that were identified on basis of the structure of Slt70 and that are indicative for the presence of a lysozyme fold (Fig. 2) (9). The degree of conservation of the boxes is, however, less in Slt35 than in other members of the family of lytic transglycosylases, that were recently identified by us in *E. coli* and other gram-negative bacteria (7, 8). The validity of this distant homology was further assessed by mutagenesis of residue Glu206 of Slt35. Substitution of this amino acid by a glutamine did affect activity, as judged by the mutant protein’s ability to break down polymeric peptidoglycan after renaturation from SDS in a zymogram analysis (Fig. 3C). Although these results indicate that Slt35 is evolutionary not closely related to Slt70, this does not necessarily imply that there are no structural similarities between both enzymes.

For crystallographic purposes, we set out to overproduce the protein in its soluble, stable form lacking the proteolysis-sensitive putative linker region. A very high degree of overproduction of protein could be achieved using the T-7 promoter system (20) (Fig. 3). The overproduced protein proved to be soluble and was shown to be able to break down polymeric peptidoglycan both in solution and in zymogram analysis (Fig. 3B).
Cloning of the gene encoding Slt35

The specific production of 1,6-anhydro-muropeptides by the overproduced protein could be demonstrated by means of HPLC and the profiles of separated muropeptides as produced by Slt70 and Slt35 were very similar (Fig. 4). Only one additional peak could be observed in the pattern as produced by Slt35. This peak probably represents a tetrasaccharide dipeptide which still contains an uncleaved glycosidic bond. Slt35 has been reported to act as an endoenzyme whereas Slt70 processes the glycan strand in an exoenzymatic way (3). This tetrasaccharide dipeptide would therefore be enriched in an Slt35 digest.

The high isoelectric point of Slt35 allowed selective binding to a strong cation exchange medium, resulting in a very efficient purification of Slt35 from the soluble fraction of a lysate, as is shown in Fig. 3A, lane 3. An additional purification step involving hydroxylapatite was applied to get rid of minor contaminating proteins. This two-step purification scheme was used to purify 100 mg amounts of Slt35 for crystallographic studies. The protein crystallized very rapidly and the resulting crystals proved to be very stable and diffracted to a resolution of about 1.8 Ångstrom (E. v. Asselt, personal communication). Further crystallographic analysis is currently underway.

It will be very interesting to see if and to what extent the structure of Slt35 resembles that of Slt70. The above mentioned lack of overall sequence homology does not necessarily exclude the possibility of structural similarity. However, the absence of the Slt70 derived fingerprints and the difference in characteristics that have been described, such as the insensitivity to bulgecin, may very well imply a totally novel structure. In either case, obtaining structure-function information on Slt35 will help to elucidate the catalytic mechanism of this whole class of enzymes, as the precise
mechanism of the transglycosylases and even that of the lysozymes is still a matter of debate (26), even though the latter mechanism is considered to be text-book knowledge.

![HPLC Analysis of 1,6-Anhydromuropeptides](image)

**FIG. 4.** HPLC analysis of 1,6-anhydromuropeptides produced by Slt35 (upper panel) and Slt70 (lower panel). The additional peak as produced by Slt35 is indicated by an arrow. The retention times of the monomeric 1,6-anhydromuramyltripeptide (Tri) and 1,6-anhydromuramyltetrapeptide (Tetra) were determined using purified standards.

**References**