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Secretion of circular proteins using sortase

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Summary

Circular proteins occur naturally and have been found in microorganisms, plants and eukaryotes where they are commonly involved in host defense. Properties of circular proteins include enhanced resistance to exoproteases, increased thermostability, longer life spans and increased activity. Using an enzymatic approach based on the bacterial sortase A (SrtA) transpeptidase, N- and C-termini of conventional linear proteins can be linked resulting in a circular protein. Circularization of bioengineered linear substrate proteins can indeed confer the desirable properties associated with circular proteins. Here we describe how cells can be manipulated to secrete circularized proteins for substrates of choice via sortase-mediated circularization in the lumen of the endoplasmic reticulum.

Keywords

Sortase (SrtA); protein circularization; intracellular sortagging; endoplasmic reticulum; secretion

1. Introduction

Circular proteins have evolved as an important component of host defense in microorganisms, plants and eukaryotes (1,2). Compared to conventional linear proteins, circular proteins display decreased sensitivity to proteolytic attack as no N- or C-termini are exposed. Circular proteins are therefore often more stable, especially in a protease-rich inflammatory milieu. In addition, circular proteins may also display improved refolding kinetics, improved stability and higher activity than their linear counterparts. Examples of naturally occurring circular proteins include the pore-forming *Enterococcus faecalis* bacteriocin AS-48 (3), *Escherichia coli* microcin J25 (MccJ25) which interferes with cell division of related species (4), plant cyclotides with antibacterial, antifungal or insecticidal properties (5–7), amatoxins and phallotoxins of lethal mushrooms (7) and the antibacterial Rhesus theta defensin-1 (RTD-1) secreted by rhesus macaque leukocytes (8–10). While the biosynthetic pathways of circular proteins remain to be deciphered in detail, most are produced by post-translational modification of linear precursors.

Synthetic methods for the production of cyclic polypeptides include modified solid phase peptide synthesis, native chemical ligation, intein-based methods and a method based on the bacterial transpeptidase sortase A (SrtA) (11,12). SrtA is an enzyme of Gram-positive

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bacterial origin involved in covalent attachment of proteins to the bacterial cell wall. Protein ligation by SrtA has been used in a number of *in vitro* protein-engineering applications (reviewed in 13). The SrtA enzyme binds to and cleaves within a C-terminal 5-residue sortase motif (LPXTG), forming an acyl-enzyme intermediate (Figure 1). This structure can be resolved by an incoming nucleophile, for example a protein with N-terminal glycines, resulting in ligation of the nucleophile to the sortase motif. Sortase-mediated circularization requires only minimal modifications of the substrate protein; a C-terminal LPXTG sortase tag and suitably exposed N-terminal glycine residue(s). Using SrtA, *in vitro* circularization of proteins such as enhanced green fluorescent protein (eGFP) (12), Cre recombinase (12), four-helix bundle cytokines interferon α 3 (IFN α 3) and granulocyte colony-stimulating factor-3 (GCSF-3) (14), human erythropoietin (EPO) (14) and the wound-healing peptide Histatin-1 (15) was accomplished. In line with the properties of naturally occurring cyclic polypeptides, the synthetic polypeptides also displayed increased stability and activity compared to their linear counterparts (12,14,15). In addition to such SrtA applications *in vitro*, we showed that the Ca^{2+} -independent *Streptococcus pyogenes* SrtA enzyme can be used for intracellular protein ligation in living cells (16). Using *S. pyogenes* SrtA, eGFP circularization could be accomplished in the cytosol and endoplasmic reticulum (ER) lumen of both *S. cerevisiae* and mammalian HEK293T cells (16).

In this chapter we describe a method for the secretion of circular proteins of choice making use of ER-luminal sortagging (see Fig. 1). The method involves the design of a linear substrate protein equipped with an N-terminal signal peptide (SP) that exposes a glycine residue after cleavage and a C-terminal sortase recognition motif (LPETG) followed by a Myc epitope tag and an ER retention signal (HDEL). The next step is transformation of *Saccharomyces cerevisiae* with the substrate protein expression plasmid and the pGAL-ER-SrtA plasmid. Sortase activity in the ER lumen will result in circularization of this substrate protein and loss of the Myc-HDEL portion of the protein. Subsequently, the circular protein advances in the secretory system and is ultimately secreted. Regulated secretion of the circular protein product is obtained by galactose-inducible ER-SrtA expression. Our method can be employed to investigate the functions of circular proteins in complex systems or for the biosynthesis of circular proteins with minimal purification.

2. Materials

2.1 Generation of circularization substrate and sortase expression vectors

1. ER-SrtA plasmid (pKS82; pRS303-pGAL-PHO5_{SP}-G-SrtA_{strep}-HA-HDEL) or TM_{Sec66}-SrtA plasmid (pKS105; pRS303-pGAL-HA-TM_{Sec66}-SrtA). Plasmids are available at Addgene (www.addgene.org). See Note 1.
2. DNA primers for PCR amplification. See Note 2.

Forward primer with 5' adaptor introducing *Xba*I-PHO5_{SP}-G-*Bam*HI:

5'-

ga**TCTAG**AATGTTTAAATCTGTTGTTTATTCAATTTTAGCCGCTTCTTTGG
CCAATGCAGGTACCATTCCCTTAG**GATCC**...(introduce gene-specific
sequence after ATG start codon)-3'

Reverse primer with 5' adaptor introducing *Sall*-LPETG-Myc-HDEL-*XhoI*:

5'-

ga**CTCGAGCCTACAGCTCGTCATGCTCGCTTGC**GGCCCCATTCAGATCCT
CTTCTGAGATGAGTTTTTTGTTCTGCGGCCGCACCAAGTTTCAGGAAGGTC
GAC...(introduce gene-specific sequence before STOP codon)-3'

3. DNA template for gene of choice (plasmid, cDNA, genomic DNA).
4. A yeast expression plasmid with a constitutive promoter, here pRS306 with *pCIT1*.
5. A high-fidelity polymerase like Phusion DNA polymerase (Finnzyme), and its specific buffer and dNTP mix.
6. A PCR purification kit, here QIAquick PCR purification kit (Qiagen)
7. Standard restriction enzymes and their related buffers, here *XbaI* and *XhoI* (New England Biolabs).
8. A gel extraction kit, here QIAquick Gel Extraction kit (Qiagen)
9. A T4 DNA ligase and its accompanying buffer (New England Biolabs).
10. *E. coli* competent cells, here DH5 α competent *E. coli* cells.
11. Sterile LB medium and LB agar plates (1% tryptone, 0.5% yeast extract, 1% NaCl).
12. Antibiotic stocks for plasmid selection (100 mg/ml ampicillin or 50 mg/mL kanamycin)
13. Nucleic acid extraction and purification kit, here QIAprep spin Miniprep kit (Qiagen).

¹The insert sequences of the sortase plasmids are the following:

Sol. ER-SrtA

ATGTTTAAATCTGTTGTTTATTCAATTTTAGCCGCTTCTTTGGCCAATGCAGGTACCATTCCTTAGGATCCAGTGTCTTG
CAAGCACAAATGGCGGCTCAGCAACTTCCTGTTATAGGGGGCATTGCCATACCAGAGCTTGGCATTAAATTTACCAATTT
TTAAAGGTTTAGGAAATACTGAGCTTATTTATGGCGCAGGAACGATGAAAGAAGAACAAAGTTATGGGAGGAGAAAAATA
ATTATCTCTTGCCAGTCATCATATTTTGGAAATTACAGGTTTCATCTCAAATGCTCTTTTCGCCGCTTGAAAGAGCACAA
AATGGGATGTCCATCTATTTAACAGATAAAAGAAAAAATTTACGAATACATCATAAAAAGATGTTTTACGGTAGCTCCTG
AACGCGTTGATGTTATCGGCGATGACAGCTGGTCTCAAAAGAAGTGAAGTGTGACTTGTACAGATATCGAAGCAACAGA
ACGTATTATTGTCAAAGGAGAACTAAAAACAGAATACGACTTTGATAAAGCGCCCGCCGATGTATTGAAAGCTTTTAAT
CATTCTTATAACCAAGTATCTACCGTCGACGCGGCCGCATACCCATACGACGTACCAGACTACGCAAATGGGGCCGCA
AGCGAGCATGACGAGCTGTAG

TMSec66-SrtA

ATGTACCCATACGACGTACCAGACTACGACAGAGACGAAATCAATCTCCGTTTATACCCCACTCATATATGTCTTTATTCT
GGTGGTGTCCCTTGATGTTTGCTTCAAGCGGATCCGAGCCAGTTAGTACAGAGAGTGTCTTGCAAGCACAAATGGCG
GCTCAGCAACTTCCTGTTATAGGGGGCATTGCCATACCAGAGCTTGGCATTAAATTTACCAATTTTAAAGGTTTAGGAA
ATACTGAGCTTATTTATCGGCGCAGAACGATGAAAGAAGAACAAAGTGTATGGGAGGAGAAAAATAATTATTCTCTTGCCA
GTCATCATATTTTGGAAATTACAGGTTTCATCTCAAATGCTCTTTTCGCCGCTTGAAAGAGCACAAAAATGGGATGTCCATC
TATTTAACAGATAAAAGAAAAAATTTACGAATACATCATAAAAAGATGTTTTACGGTAGCTCCTGAACGCGTTGATGTTA
TCGATGATACAGCTGGTCTCAAAGAAGTGAAGTGTGACTTTAGTGAAGTGTACAGATATCGAAGCAACAGAACGTATTATTGTCAA
AGGAGAACTAAAAACAGAATACGACTTTGATAAAGCGCCCGCCGATGTATTGAAAGCTTTTAATCATTCTTATAACCA
GTATCTACCTAG

²The termini of most proteins are unstructured and accessible for modification by SrtA. The proximity of N- and C-terminus depends on the protein of choice and can be studied when a crystal structure is available. A longer linker sequence can be introduced when it is expected that the termini are in close proximity. We have found that also termini that are buried within a structure can be accessible for sortagging like in the case of UCHL3 [12] and HUWE1 (unpublished results).

14. Standard equipment, consumables and chemicals for routine molecular biology techniques including PCR amplification of DNA fragments, DNA separation and visualization, UV spectrophotometry and *E. coli* culturing.
15. DNA sequence primers

2.2 Plasmid isolation

1. Bacterial stock (typically *E. coli* DH5 α) transformed with the substrate or sortase plasmids.
2. Sterile LB medium and LB agar plates (1% tryptone, 0.5% yeast extract, 1% NaCl).
3. Antibiotic stocks for plasmid selection (100 mg/mL ampicillin or 50 mg/mL kanamycin)
4. High-speed plasmid Maxi kit (QIAGEN)

2.3 Yeast transformation with substrate and sortase plasmids

1. All yeast reagents are listed in Table 1.
2. Freshly plated auxotrophic *S. cerevisiae* strain, here W303-K700
3. Salmon sperm carrier DNA (Sigma-Aldrich), store at -20°C .
4. 50% polyethylene glycol 4000, stable at room temperature.
5. 1 M lithium acetate, stable at room temperature.
6. YPDA medium: 1% yeast extract, 2% bacto peptone, 2% glucose, 1 $\times$ adenine, stable at room temperature.
7. 100 $\times$ Adenine, Uracil, Tryptophan, Leucine, Histidine amino acid solutions, stable at room temperature.
8. Selection plates lacking the amino acid selective marker(s).

2.4 Galactose induction to initiate sortase expression and substrate circularization

1. *S. cerevisiae* isolates transformed with the substrate and sortase plasmids.
2. YNB 2% glucose medium: 1 $\times$ YNB, 2% glucose.
3. YNB 2% glucose medium: 1 $\times$ YNB, 2% glucose.
4. 100 $\times$ Adenine, Uracil, Tryptophan, Leucine, Histidine amino acid solutions, stable at room temperature.
5. YPGA medium: 2% bactopectone, 1% yeast extract, 2% galactose and 1 $\times$ adenine.

2.5 Detection of circular protein

1. Optional: antibody for immunoprecipitation.
2. Optional: 100% trichloroacetic acid (TCA) solution for protein precipitation.
3. Optional: Amicon Ultra Centrifugal Filters (Millipore) for protein concentration.

4. Equipment and materials for SDS-PAGE gels and Coomassie staining.
5. MS/MS facilities for sample analysis.

3. Methods

3.1 Generation of circularization substrate and sortase expression vectors

1. Prepare the PCR reaction mix on ice: typically 1–10 ng of the template DNA, 5 μ l each of the 10 μ M stock solution of the forward and reverse primers, 10 μ l of 5 $\times$ PCR buffer, 5 μ l of dNTP mixture (2.5 mM each), 1 U of high fidelity Phusion polymerase, and sterile water to a final volume of 50 μ l.
2. Run the PCR reaction in a thermocycler with a 25-cycle protocol alternating 15 s at 98°C, 15 s at 56°C and 1 min at 72°C.
3. Pipette 5 μ l of the PCR reaction, add DNA loading dye and load the mixture on a 1% agarose gel to analyze the amplified product. *See Note 3.*
4. Purify the remaining 45 μ l of the substrate PCR reaction using the protocol detailed in the PCR purification kit.
5. In individual eppendorf tubes, cut the amplified DNA fragment coding for the circularization substrate and the expression vector of choice (pRS306 or other) with the appropriate restriction enzymes, in this case *Xba*I and *Xho*I. Typically, 1 μ g of plasmid DNA is used.
6. Load the digestion products on a 1% agarose gel and following separation extract the cut insert and vector fragments separately. *See Note 4.*
7. Isolate the DNA from the gel pieces using the protocol detailed in the QIAquick Gel Extraction kit.
8. Prepare the ligation reaction with a 5:1 ratio of insert:plasmid. Always include a control ligation reaction without the insert and a control reaction with T4 DNA ligase. Add 1 U of T4 DNA ligase together with the ligase buffer and add sterile water to a final volume to 20 μ l.
9. Incubate the ligation reaction 1–2 hours at room temperature or overnight at 16°C (preferred). *See Note 5.*
10. For bacterial transformation, add 10 μ l of the ligation mixture to 50 μ l of DH5 α competent cells. Incubate on ice for 30 min to allow for DNA uptake.
11. Heat-shock the cells for 45 s at 42°C and immediately transfer the tubes on ice.

³Problem solving in case the PCR reaction does not yield a product: the template concentration might be too low (especially when cDNA is used). Increase the number of cycles from 25 to 35. When increasing the number of cycles does not yield a product, a two-step PCR protocol can be performed. First step: perform the primary PCR with the long primers for 5 rounds. Second amplification step: add short primers (covering the 5' ends of the long primers) to the reaction and perform an additional 25 rounds.

⁴Before and during cutting of the fragments from the 1% agarose gel, use low wavelength UV irradiation as high wavelength can introduce DNA mutations.

⁵The 20 μ l ligation reaction can be split in two: 10 μ l can be incubated for 1–2 hours at RT, the other 10 μ l of the reaction can be incubated overnight at 10°C.

12. Add 1 mL of LB medium without antibiotics and let the cells regenerate in the shaker for 1 h at 37°C.
13. Spread 100 µl of the transformation mixture on LB agar plates supplemented with ampicillin or kanamycin and incubate overnight at 37°C. The remainder of the transformed bacteria can be stored at 4°C for up to 1 week.
14. The following day, pick colonies and use to inoculate 2 mL LB supplemented with ampicillin. Grow the cultures overnight in the shaker at 37°C.
15. Purify the plasmid DNA of each clone using the plasmid purification kit following the manufacturer's instructions.
16. Take 2 µl of each plasmid preparation and perform restriction digest analysis using the *Xba*I and *Xho*I enzymes to confirm the presence of the insert.
17. Final check the insert by DNA sequencing and select a plasmid containing the correct insert sequence. *See* Note 6.
18. Store the plasmid at -20°C.

3.2 Plasmid isolation

1. Transform DH5α competent cells with the correct substrate plasmid and the obtained ER-SrtA and TM_{Sec66}-SrtA plasmids.
2. Isolate DNA by following the protocol detailed in the High-speed plasmid Maxi kit.

3.3 Yeast transformation with substrate and sortase plasmids

1. Aseptically pick a single *S. cerevisiae* colony from a freshly streaked YPD agar plate in to 5 mL of YPD medium with 1× adenine and culture overnight in the shaker at 30°C.
2. The next day, dilute the culture to OD₆₀₀ = 0.2 in 50 ml YPD + A medium. Incubate in the shaker at 30°C until OD₆₀₀ = 0.4–0.7 (this will take about 4 hours).
3. Spin down at 3000 rpm for 5 min. Wash the pellet with 25 ml H₂O, spin at 3000 rpm for 5 min. Resuspend in 1 ml 0.1 M LiAc and transfer to an eppendorf tube.
4. Spin down at 3000 rpm for 5 min and aspirate the supernatant. Take the pellet up in 400 µl of 0.1 M LiAc (total volume ~500 µl).
5. Setup tubes for transformation with the substrate plasmid, the sortase plasmid and the combined plasmids. *See* Note 7.
6. For each transformation, aliquot 50 µl of the cell suspension in an eppendorf tube. Spin down and remove the LiAc.
7. Add 360 µl of TRAFO mix to each cell pellet.

⁶For cloning and expression of other substrate(s), the *Bam*HI and *Sal*I restriction sites can be used bypassing the need for long primers.

⁷Always setup a transformation with the single plasmids as a control for unmodified substrate and sortase expression.

8. Incubate with rotation at 30°C for 30 min.
9. Heat shock at 42°C for 15 minutes. *See* Note 8.
10. Spin down and resuspend the pellet in 100 µl H₂O.
11. Plate on appropriate selective YNB 2% glucose plates (-His/-Ura/-Leu/-Tryp) and grow for 2–3 days at 30°C.
12. Restreak single colonies on fresh selective plates and grow for 2–3 days at 30°C. *See* Note 9.
13. Pick single colonies to make glycerol stocks and/or do the experiment.

3.4 Galactose induction to initiate sortase expression and substrate circularization

1. Inoculate a yeast colony in 25 ml of YNB 2% glucose medium with necessary amino acids (add Leu, Trp, Ade, His, Ura, with exception of selection markers on transformed plasmids). Grow overnight in the shaker at 30°C.
2. Next day late afternoon, inoculate the culture in 25 ml YNB 0.3% glucose + amino acids. Grow for about 16 hours in the shaker at 30°C.
3. Next day morning, inoculate cells in YPGA medium at an OD₆₀₀ of 0.2.
4. Harvest supernatant and cell pellets after 2, 4 and 8 hours. The circular protein is secreted in the media.

3.5 Detection of circular protein

1. If an antibody is available for the substrate of choice, the circular protein can be isolated from the media by immunoprecipitation. Otherwise, precipitate the protein fraction from the media by TCA precipitation or concentrate the protein fraction using spin columns (for example Amicon Ultra Centrifugal Filters from Millipore).
2. Analyze the immunoprecipitate or protein fraction by SDS-PAGE gel and immunoblotting and/or Coomassie staining. The circular protein usually runs at a lower molecular weight compared to its linear counterpart. In addition, the linear substrate protein contains a Myc tag that is lost upon circularization.
3. Cut the area containing the circular protein from the Coomassie-stained gel and perform MS/MS analysis to confirm the molecular weight of the circularized protein.

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⁸The timing of the heat shock treatment is important.

⁹Restreaking is important as amino acid selection is not lethal and non-transformed cells might still be present. Try to restreak the colonies so that one ends up with single colonies. A tree-shape is commonly used.

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Sortase reaction:

Secretion of circular protein:

Figure 1. Secretion of circular proteins using sortase-mediated protein ligation in the endoplasmic reticulum

Left: The sortase protein-ligation reaction. A substrate protein is equipped with a LPXTG sortase recognition motif followed by an epitope tag of choice. Sortase (SrtA) binds to the LPXTG motif resulting in formation of an acyl-enzyme intermediate and release of the G-epitope tag fraction of the substrate protein. A nucleophile protein with an Nterminal glycine can resolve the acyl-enzyme intermediate thereby forming the ligation product through a regular peptide bond. Right: Secretion of circular protein using ERluminal sortagging. A substrate protein with an N-terminal Pho5 signal peptide (SP) and C-terminal LPETG-Myc-HDEL motif is expressed in the cytosol. The Pho5 SP induces import into the ER (1) and the HDEL motif prevents the substrate protein from exiting the ER. Cleavage of the Pho5 signal peptide exposes an N-terminal glycine (G). Induction of soluble ER-SrtA or transmembrane-bound TM_{Sec66}-SrtA initiates the protein ligation reaction that forms the circular protein (2). The loss of the Myc epitope tag can be used as a read-out for successful substrate recognition by the SrtA enzyme. The circularized protein no longer contains the HDEL ER retention signal and therefore enters the secretory pathway resulting in secretion.

Table 1

Yeast solutions and media

<i>S. cerevisiae</i> strain W303-K700	<i>MATalpha, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+</i>
YP	10g yeast extract and 20g bacto peptone in 900 ml deionized water, autoclave and cool down
20% glucose	20 g/100 ml, filter sterilize
20% galactose	20 g/100 ml, filter sterilize
10x YNB	134 gram yeast nitrogen base with ammonium sulfate without amino acids/1 L, filter sterilize
100x Adenine	0.55 g/100 ml, filter sterilize
100x Uracil	0.224 g/100 ml, filter sterilize
100x Tryptophan	0.8 g/100 ml, filter sterilize, protect from light
60x Leucine	1.31 g/100 ml, filter sterilize
100x Histidine	2.09 g/100 ml, filter sterilize, protect from light
1 M LiAc	6.6 g Lithium Acetate/100 ml, autoclave
0.1 M LiAc	0.66 g/100 ml, autoclave
50% PEG	50 ml polyethylene glycol 4000/100 ml, autoclave
TRAFO mix	240 µl 50% PEG, 36 µl 1.0 M LiAc, 10 µl salmon sperm carrier DNA, 1 µg, H ₂ O upto 360 µl for each transformation
YPDA	To 900 ml YP stock medium, add 100 ml 20x glucose and 10 ml 100x Adenine
YPGA	To 900 ml YP stock medium, add 100 ml 20x galactose and 10 ml 100x Adenine
YNB 2% glucose	20 g/900 ml D-glucose, autoclave and cool down, add 100 ml 10x YNB
YNB 0.3% glucose	3 g/900 ml D-glucose, autoclave and cool down, add 100 ml 10x YNB
Selection plates	4 g D-glucose and 4 g agar in 180 ml, autoclave and cool down, add 20 ml 10x YNB and all amino acids (adenine, uracil, tryptophan, leucine, histidine) except for the selection marker(s) of the transformed plasmids