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# Peptide oligomers from ultra-short peptides using sortase

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ABSTRACT

Sortase A catalyzed ligation of ultra-short peptides leads to inter/intra-molecular transpeptidation to form either linear or cyclic oligomers dependent upon the peptide length. Cyclic peptides were the main products for peptides with more than 15aa. However, for ultra-short (< 15aa) peptides, cyclic oligomers became predominant in prolonged reactions. Peptides with 1–3 aminoglycines were equally active but peptide oligomers from peptide containing more than one aminoglycine were prone to hydrolysis.

## 1. Introduction

Bioactive peptides have many unique and unbeatable features in comparison with proteins and other synthesized polymers. Bioactive peptides regulate many physiological processes, acting at some sites as endocrine or paracrine signals and at others as neurotransmitters or growth factors [1], and show useful properties for human health, including antimicrobial, antifungal, antiviral, and antitumor activities [2,3]. In addition, peptides can interact specifically with toxic metal ions to act as effective chelating agents [4,5], and peptides with specific sequences can self assemble to form materials with regular nanostructures [6–8]. Although most peptides can be produced using the molecular biology approach, they are produced in limited quantities at high costs and associated with some difficulties in purification and isolation. In addition, some peptides with special structures, such as cyclic peptides, can hardly be produced by the biological method. This is especially true for the biomedical applications in which long peptides with functional sequences are usually needed [8].

*Staphylococcus aureus* Sortase A ($SrtA_{Staph}$), a cysteine transpeptidase, recognizes an LPXTG motif near C-terminus of a protein substrate and cleaves the peptide bond between threonine and glycine residues to form a thioacyl enzyme intermediate between the catalytic cysteine and the substrate threonine [9,10]. The intermediate reacts with the N-terminus of an oligoglycine in nucleophilic substitution with formation of amide bond between the substrate threonine and the incoming glycine [11]. This $SrtA_{Staph}$-catalyzed transpeptidation reaction has been used for protein/peptide ligation and labeling [12,13-17,26,27]. Recently, Wu et al. found that Sortase A mediated transpeptidation seemed to be length dependent for peptides with 16–19 amino acids [25]: a cyclic dimer was the major product for peptides with 16 and 17 amino acids while the main product of 19aa peptide was a cyclic monomer. However, $SrtA_{Staph}$-mediated peptide ligation and thus peptide oligomer production using ultra-short (< 15 amino acids) peptides has not been studied.

## 2. Experimental details

### 2.1. Peptides

All peptides for this study (> 90% in purity) were synthesized by Genescript Corp. Methyl ester modified peptides (peptide-OMe) were produced according to method reported previously [18]. The replacement of the glycine in the minimal $SrtA_{Staph}$ recognition sequence, LPRT G, by the threonine methyl ester (-OMe) was shown to minimize product hydrolysis [18].

### 2.2. Sortase a expression and purification

Wild type *Staphylococcus aureus* Sortase A containing residues 60–206 (St-$SrtA_{\Delta 59}$), named as $SrtA_{Staph}$, was produced in *Escherichia coli* (*E. coli*). Plasmid pET15bSt-$SrtA_{\Delta 59}$ (a gift from Dr. Robert Clubb, UCLA) was used for expression. pET15bSt-$SrtA_{\Delta 59}$ was transformed into BL21-Gold(DE3) competent cells (Stratagene, CA). Cultures were grown in 1.0 L of Luria-Bertani (LB) media supplemented with ampicillin (200 μg/mL) at 37 °C with shaking at 250 rpm. After cells were grown to an optical density of 0.6 at 600 nm, 1 mM isopropyl thioglucoside (IPTG, Fisher Scientific) was added to induce protein expression. The expression continued for 3 h at 37 °C. The cells were harvested by centrifugation at 4,000 rpm. Cell pellets were resuspended in the buffer B (20 mM Tris-HCL, pH 7.5, 150 mM NaCl, 15 mM 2-mercaptoethanol)

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and lysed by sonication. $SrtA_{Staph}$ protein bearing N-terminal His tag was purified by affinity chromatography using a His-Select Nickel Affinity gel (Sigma). Purified protein was eluted with buffer B containing 200 mM imidazole and then concentrated using Amicon Ultra Centrifugal filters (Millipore) with MWCO of 10 kDa. $SrtA_{Staph}$ with good reactivity was obtained.

### 2.3. Sortase FRET activity assay

Sortase A substrate Dabcyl-LPGTG-Edans (Anaspec, CA) was dissolved in the reaction buffer (300 mM Tris-HCl, pH = 7.5, 150 mM NaCl, 5 mM $CaCl_2$) and added at a final concentration of 10 μM. Peptide cleavage was monitored as an increase in fluorescence intensity over time at 460 nm ( $\lambda_{ex}$ = 360 nm) with a SynergyMx spectrometer (BioTek Instrument, VT). Reaction was carried out in sortase activity buffer in a volume of 100 μL at 25 °C with 12 μM $SrtA_{Staph}$, and 1 mM triglycine.

### 2.4. Sortase-mediated peptide oligomer synthesis

Reactions were carried out by combining 250 μM methyl ester peptides and 12 μM $SrtA_{Staph\ in}$ sortase buffer (300 mM Tris-HCl, pH = 7.5, 150 mM NaCl, 5 mM $CaCl_2$) and incubated at 37 °C for times indicated. At each time point, 20 μL aliquots were withdrawn, and reaction was quenched by adding 5 μL of 0.1% trifluoroacetic acid (TFA). The reaction aliquots were purified through $ZipTip_{C\text{-}18}$ column (Millipore) and loaded on the target plate for MALDI-TOF/TOF analysis.

### 2.5. MALDI –TOF/TOF MS

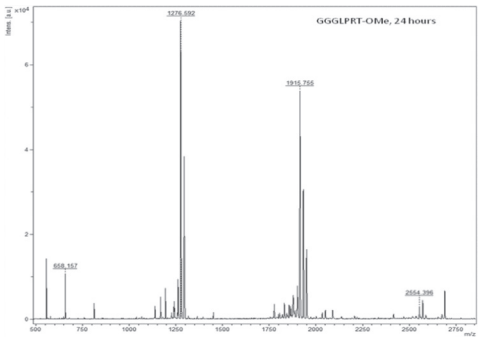
Peptide products in the reaction mixtures were analyzed via Matrix-Assisted Laser Desorption Ion-Time of Flight/Time of flight (MALDI-TOF/TOF) mass spectrometry. All MALDI mass spectra were acquired using a Bruker UltraFlextreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics). The instrument was used in reflector mode and acquisition was optimized for a mass range from 600 to 3000 Da. All measurements were done with constant laser power of 40. The matrix used was α-cyano-4-hydrocinnamic acid (10 mg/mL in 50% acetonitrile with 0.05% trifluoroacetic acid). Experiments were carried with fixed amount of exogenous internal standard (neurotensin, monoisotopic MW 1671.9) [19]. A total of 4500 laser shots were summed for each spectrum (each sample spot was irradiated at 9 random but evenly distributed locations with 500 shots per location). Relative signal intensities within MALDI mass spectra were compared to determine relative yield of a particular peptide product. Using intensities of peaks that correspond to reaction products, yields were estimated as, for example, $\%\ \text{monomer} = I_{monomer}/(I_{monomer} + \Sigma I_{dimeric\ species} + \Sigma I_{trimeric\ species} + \Sigma I_{tetrimeric\ species})$, where I is intensity. The values for tetramers were also added when applicable.

### 2.6. CD analysis

The CD spectra of peptides were recorded on Jasco J-710 spectropolarimeter. The CD spectra were scanned at 20 °C in a capped, quartz optical cell with a 1.0 mm path length. Data was collected from 250 to 190 nm at an interval of 1.0 [21] nm with an integration time of two seconds at each wavelength. Five to ten scans were averaged, smoothed and background-subtracted for each measurement.

### 2.7. Peptide self-assembly

Peptides were dissolved in 300 mM Tris-HCl buffer (pH = 7.5) containing 150 mM NaCl and 5 mM $CaCl_2$. Peptide solutions (250 μM) were applied onto the surfaces silicon wafers and incubated in a moisture environment at 37 °C overnight. After washing with deionized water, peptide samples on silicon wafers were coated with gold. Morphologies of peptide aggregates were studied using scan Auriga Modular CrossBeam workstation (Carl Zeiss Inc., Thornwood, NY). Formation of peptide aggregates in solution was estimated using a fluorescence probe 1-anilinonaphthalene-8-sulfonic acid (1,8-ANS) [20]. ANS (20 μM) fluorescence emission spectrum increase caused by peptide aggregration was recorded on a fluorescent Microplate Reader (Biotek Inc.) by setting excitation wavelength at 369 nm.




| Peptides | Yield (%) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Monomer | | Dimer | | Trimer | | Tetramer | | Pentamer | |
| | Lin | Cyc | Lin | Cyc | Lin | Cyc | Lin | Cyc | Lin | Cyc |
| GGGLPRT | - | - | 15 | 43 | 18 | 21 | - | - | - | - |
| GGLPRT | - | - | - | - | 7 | 62 | 5 | 25 | - | - |
| GLPRT | - | - | - | - | - | 32 | 9 | 47 | 5 | 11 |

**Fig. 1.** $SrtA_{Staph}$ mediated ligation and cyclization of short peptides containing one to three N-terminal glycines (Lin- Linear peptide/oliomer; Cyc-Cyclic peptide/oligomer). Top, typical MALDI TOF/TOF MS spectra from GGGLPRT-OMe; Bottom, calculated yields of different products. Reaction time: 24 h.

## 3. Results and discussions

We started our study on $SrtA_{Staph}$-mediated ultra-short peptide ligation using the minimal $SrtA_{Staph}$ recognization sequence based on publications, GGGLPRT-OMe, with a methyl ester at the C-termini. $SrtA_{Staph}$-catalyzed small peptide ligation was very efficient and 97% of GGGLPRT-OMe was converted into peptide oligomers at the end of 24 h reaction (Fig. 1). Resulting peptides were a mixture of dimers, trimers, tetramers, and pentamers in linear and cyclic forms, with dimer and trimer peptides as the main products.

Kinetic studies revealed that linear peptide oligomers formed early and rapidly (Fig. 2). Significant amounts of linear dimer, trimer, and tetramer were found after one hour of reaction when very low concentrations of corresponding cyclic oligomers were detected. However, the amount of cyclic oligomers increased as the reaction time was extended. There was a good dynamic correlation between linear oligomer disappearance and cyclic oligomer formation over the reaction period of 0–5 h. These data show that $SrtA_{Staph}$-catalyzed small peptide ligation includes two reactions: 1) formation of linear oligomers through the head-to-tail peptide ligation, and 2) cyclization of linear oligomers to form cyclic products by followed intramolecular transpeptidation. Interestingly, despite the quick accumulation of linear oligomers at the beginning of the reaction, linear oligomers in hydrolyzed forms (without methyl ester at the C-termini) were maintained at relatively stable levels within the first five hours of reaction. Increase of linear oligomers in hydrolyzed form was observed after a long time (> 5 h) reaction. There was good correlations between the formation of cyclic peptides and the production of corresponded linear peptides in the hydrolyzed forms, indicating the presence of $SrtA_{Staph}$-mediated ring opening reactions in cyclic oligomers. Oligomers containing more peptide units were especially prone to $SrtA_{Staph}$ mediated hydrolysis, and all GGGLPRT tetramers (both cyclic and linear) formed at the beginning of reactions disappeared at the end of 24-h reaction (Fig. 1).

It is known that the polyglycine sequence at the N-terminus of the