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Evaluation of P1′ substrate specificity of staphylococcal SplB protease*

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***Staphylococcus aureus* is a dangerous human pathogen characterized by growing antibiotic resistance. Virulence of *S. aureus* relies on a variety of secreted and cell surface associated virulence factors among which certain proteolytic enzymes play an important role. Amid staphylococcal extracellular proteases, those encoded by the *spl* operon remain poorly characterized, both in terms of enzymology and their physiological role. Initial data demonstrated that Spl proteases exhibit restricted substrate specificity. This study describes development of convenient protein FRET substrates for SplB protease and characterization of the substrate preference of the protease at the P1′ position. Kinetic data on hydrolysis of a panel of substrates substituted at the said position is provided.**

Key words: serine protease, serine protease-like, SplB, *Staphylococcus aureus*, substrate specificity

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INTRODUCTION

Nearly 30% of the human population is transiently or permanently colonized by *S. aureus* with no visible symptoms. At the same time, this bacterium is a common, dangerous, opportunistic pathogen, responsible for approximately 15% of hospital infections worldwide and a substantial share of community acquired infections. *S. aureus* causes relatively harmless skin infections such as abscesses, boils, impetigo or purulent wounds (Noble, 1998), but is also responsible for internal infections characterized by a high mortality rate: endocarditis, pneumonia, meningitis, arthritis, and others (Archer, 1998).

Staphylococci secrete multiple proteases which, as a group, constitute important virulence factors of these bacteria (Dubin, 2002; Kolar *et al.*, 2013). Proteases of the *spl* operon are currently the least characterized in terms of their role in virulence. *Spl* operon encodes from two up to six proteases designated SplA-F, depending on the strain. Location of the operon at the staphylococcal pathogenicity island and sequence homology with V8 protease and epidermolytic toxin suggests involvement in virulence, but no direct evidence has been provided as yet.

Our previous biochemical and structural characterization of the SplB protease (Dubin *et al.*, 2008) provided interesting data which distinguished this enzyme from among homologous serine proteases of the S1 family (Rawlings *et al.*, 2010). Of significance for the current work, restricted substrate specificity of SplB was demonstrated. S1 family serine proteases usually exhibit strong substrate preference at the P1 position (Laskar *et al.*, 2012), whereas the consensus sequence recognized and cleaved by SplB extends to 4 consecutive residues at the nonprime side of the cleavage site (WELQ*; asterisk indicates the cleavage site). We also demonstrated that the enzyme recognizes P1′ residue, although with less specificity. The purpose of the current work was to provide quantitative kinetic data characterizing the substrate preference of SplB protease at the P1′ substrate position and thus confirm and strengthen previous observations. To this end we devised a fluorescence quenched protein substrate for SplB, generated a panel of its variants substituted at the P1′ position and evaluated the hydrolysis kinetics by the SplB protease as reported below.

MATERIALS AND METHODS

Construction of expression vectors. Genes encoding enhanced cyan and enhanced yellow fluorescent proteins were PCR amplified from pECFP-N1 and pEYFP-N1 (Clontech), respectively. Primers used for amplification contained NcoI and BamHI sites which were used for cloning of both genes into pET28a (Novagen) resulting in pET28a_cfp and pET28a_yfp, respectively. The reverse primer used for cloning *cfp* contained an additional sequence coding for a polypeptide linker — GSWELQGS. For construction of CFP-GSWELQGS-YFP fusion protein expression vector, *yfp* was PCR amplified from pET28a_yfp and cloned into pET28a_cfp using XhoI and BamHI. The length of the polypeptide linker was further modified by inserting additional Gly-Ser repeats by site directed mutagenesis of pET28a_cfp_yfp. Plasmids encoding fusion proteins containing different P1′ residues were also prepared by site directed mutagenesis. All plasmids used in this study were sequenced to confirm the presence of desired insert modifications, and to ensure that no random nsucleotide changes were introduced during manipulations.

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Abbreviations: CFP, cyan fluorescent protein; FRET, Förster resonance energy transfer; GST, glutathione S-transferase; Spl, serine protease-like; YFP, yellow fluorescent protein

Protein expression and purification. All variants of CFP-YFP fluorescence-quenched fusion protein substrate were expressed with six histidine tag in *E. coli* BL21(DE3). Bacteria were cultured at 37°C until OD_{600} reached 0.6. Expression of recombinant protein was induced with 1 mM IPTG, the temperature was decreased to 22°C and the culture continued overnight. Cells were collected by centrifugation and lysed by sonication in 50 mM sodium phosphate pH 8.0 containing 300 mM NaCl and 10 mM imidazole. Recombinant protein was recovered with Chelating Sepharose (GE Healthcare) and dialyzed overnight against 50 mM Tris/HCl pH 8.0 to obtain samples of ~90% purity as assessed by SDS/PAGE. Such protein substrates were stored frozen until further use.

Activity assay and determination of enzyme kinetics. Different variants of FRET protein substrate based on SplB consensus sequence were evaluated for SplB catalyzed hydrolysis at 37°C in 50 mM Tris/HCl pH 8.0. Enzymatic activity was monitored as an increase in fluorescence at λ_{ex}=440 nm, λ_{em}=485 nm and 528 nm. All measurements were performed at least in triplicates.

The fusion protein (1 μM) half-life was determined upon incubation with 10 nM SplB under conditions described below. k_{cat}/K_m values were determined under pseudo-first-order conditions in which the substrate concentration is far below the estimated K_m. Fluorescent protein substrates (1 μM; Exact value of K_m was not determined due to problems with substrate solubility, but was estimated at above 100 μM.) were incubated in the presence of increasing enzyme concentrations (25 nM, 50 nM, 100 nM, 200 nM and 500 nM) and changes in fluorescence intensity (Int) were recorded for 60 min. Using OriginPro software, progress curves described by the equation $Int = I_0 + I_{max}(1\text{-}\exp(-[E]\ k_{cat}/K_m\ t))$ were fitted to experimental data. In the formula above, I_0 represents the initial fluorescence of the uncleaved substrate, I_{max} the maximum fluorescence intensity (fully hydrolyzed substrate), and $[E]$ is the total enzyme concentration.

RESULTS AND DISCUSSION

Generation and optimization of a fluorescence quenched protein substrate for SplB protease

A Förster Resonance Energy Transfer (FRET) protein substrate for SplB protease was developed by creating a fusion of CFP and YFP (Felber, Cloutier *et al.* 2004), and introducing SplB consensus sequence into the linker connecting the two fluorescent proteins. The CFP-GSWELQGS-YFP fusion protein was produced in *Escherichia coli* BL21(DE3) and purified by nickel affinity chromatography as described in Materials and Methods with a yield of ~20 mg of purified protein per 1 L starting culture. When CFP-GSWELQGS-YFP was excited at 440 nm wavelength, two emission peaks were observed at 485 nm and 528 nm. These corresponded to emission peaks of CFP alone (major peak at 485 nm and minor at 528 nm) and YFP alone (a single peak at 528 nm) (Fig. 1A). For a CFP-GSWELQGS-YFP fusion, the fluorescence intensity at 528 nm was higher than that

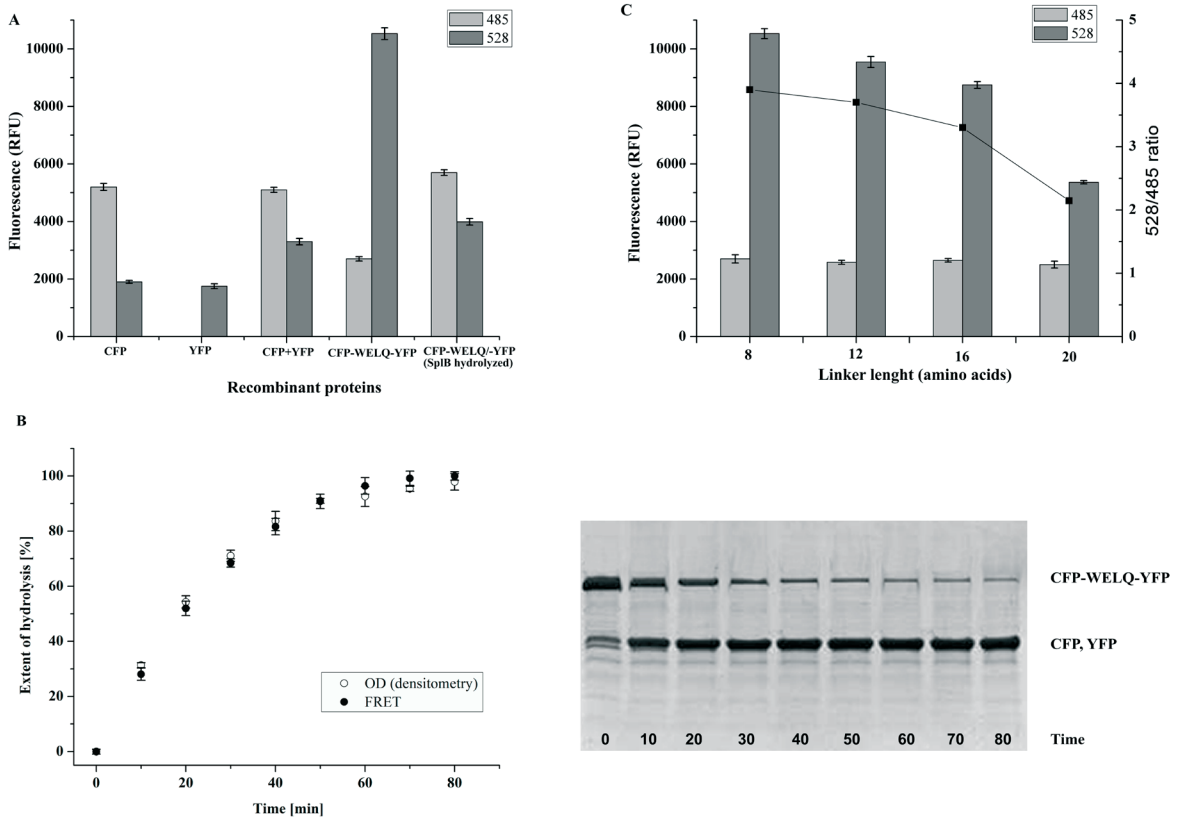

Figure 1. Basic properties of protein FRET substrates for SplB protease.
(**A**) Fluorescence intensity at indicated wavelengths upon excitation at 440 nm. (**B**) Hydrolysis of CFP-GSWELQGS-YFP by SplB protease monitored by SDS/PAGE and densitometry or using FRET. Correlation between densitometric and fluorometric measurements is immediately visible (graph). (**C**) Effect of the linker length on FRET efficiency. Black squares indicate a ratio between emission intensity at 528 nm and 485 nm used as a measure of extent of the substrate hydrolysis.

Table 1. Comparison of P1′ substrate specificity of SplB protease determined using two types of fusion protein substrates.
The kinetics of hydrolysis of GST-WELQ↓X-Staphostatin A substrates determined previously and expressed as substrate half-life under given conditions (Dubin *et al.,* 2008) are compared to that of CFP-GSWELQ↓XS-YFP determined in this study (expressed both as substrate half-life at conditions analogous to previous study and k_{cat}/K_m values).

P1′ residue (X)	GST-WELQ↓X-Staphostatin A	CFP-GSWELQ↓XS-YFP	
	Activity normalized to the best hydrolyzed substrate ($t_{1/2}$, min)		k_{cat}/K_m [$M^{-1}s^{-1}$]
Gln	100% (35)	100% (25)	5544 ± 115
Asn	86% (40)	71% (35)	3960 ± 95
Gly	78% (45)	56% (45)	3080 ± 61
Ala	nd	50% (50)	2772 ± 73
Glu	54% (65)	36% (70)	1980 ± 45
Lys	18% (210)	13% (200)	693 ± 25
Leu	14% (250)	10% (260)	533 ± 15
Met	13% (280)	9% (280)	495 ± 13
Phe	10% (360)	6% (400)	346 ± 18

of an equimolar mixture of CFP and YFP indicating FRET. Incubation of CFP-GSWELQGS-YFP with SplB resulted in gradual decrease of fluorescence intensity at 528 nm and increase of fluorescence at 485 nm. The extent of both effects corresponded to hydrolysis of the fusion protein into polypeptides corresponding to CFP and YFP as monitored by SDS-PAGE and densitometry (Fig. 1B). Therefore, throughout the study a ratio between emission intensity at 528 nm and at 485 nm was used as a measure of the extent of substrate hydrolysis.

Once the FRET substrate of SplB protease was initially established, the length of the linker was optimized to ensure a compromise between steric accessibility of the consensus sequence and FRET efficiency. To this end, four fusion proteins with the peptide linker of 8, 12, 16 and 20 amino acids were tested. In each case, the consensus sequence (WELQ) was located in the middle part of the inker and flanked by $(Gly\text{-}Ser)_n$ repeats. Increase in the linker length resulted in decrease of FRET efficiency, up to 50% for a 20 amino acid linker compared to the 8 amino acid linker (Fig. 1C). Since the increase in the linker length had no influence on the rate of hydrolysis of the fusion protein by SplB, 8 amino acid long linker was chosen for further studies.

Reevaluation of P1′ substrate specificity of SplB

FRET protein substrates based on the SplB consensus sequence were used for assessing the P1' substrate specificity of the protease by analyzing the kinetics of hydrolysis of substrate variants substituted at the tested position with different amino acid residues. P1' substrate preference of SplB was previously assayed using a number of recombinant protein substrates containing two globular fusion partners (GST and staphostatin A) connected by a linker containing tested cleavage sties of a following structure: WELQ↓X, where X denotes the tested P1' amino acid (Dubin *et al.*, 2008). To evaluate to what extent these results were substrate specific we determined the CFP-GSWELQ↓XS–YFP protein half-lives in the presence of SplB exactly as has been done previously for GST-WELQ↓X-Staphostatin A substrates (Table 1). The P1' substrate preference of the SplB protease determined with both classes of substrates is identical, demonstrating that the obtained results are not substrate specific. SplB prefers amino acids with polar, uncharged side chains, that is Gln and Asn at P1' substrate position. Residues containing small side chains (Gly, Ala) are also accepted. Contrary, bulky, hydrophobic side chain containing amino acids (Phe, Met) are the least preferred of all tested residues.

Apart from semi-quantitative assessment of the rate of hydrolysis, the fluorescence-quenched substrates devised in this study allowed quantitative characterization of kinetics of SplB catalyzed hydrolysis of P1' substituted CFP-GSWELQ↓XS-YFP substrates (Table 1). The P1' substrate preference, measured as k_{cat}/K_m, corresponds to the preference given as substrate's half-life at the conditions of our experiment. The difference in k_{cat}/K_m values between most efficiently hydrolyzed and the least efficiently hydrolyzed of the substrates tested was over 16 fold.

Majority of the S1 family serine proteases exhibit strong preference for the P1 residue. The preference at other positions is usually more relaxed and due to this assumption it is rarely determined experimentally. Our previous study demonstrated strong preference of SplB protease at the non-primed sites (P4-P1) and less, but still pronounced preference at P1'. Here, we developed SplB substrates allowing convenient determination of kinetics of hydrolysis and reevaluated the P1' preference of SplB. Our study confirmed the previously determined P1' preference of SplB and provided quantitative kinetic data. Moreover, our study provided convenient FRET substrates for the SplB protease and created a platform for easy future modification and testing of different substrate sequences.

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