Structure-activity studies of *Streptococcus pyogenes* enzyme SpyCEP reveal high affinity for CXCL8 in the SpyCEP C-terminal

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Abstract

The *Streptococcus pyogenes* cell envelope protease (SpyCEP) is vital to streptococcal pathogenesis and disease progression. Despite its strong association with invasive disease, little is known about enzymatic function beyond the ELR^+ CXC chemokine substrate range. As a serine protease, SpyCEP has a catalytic triad consisting of aspartate (D151), histidine (H279), and serine (S617) residues which are all thought to be mandatory for full activity. We utilised a range of SpyCEP constructs to investigate the protein domains and catalytic residues necessary for enzyme function. We designed a high-throughput mass spectrometry assay to measure CXCL8 cleavage and applied this for the first time to study the enzyme kinetics of SpyCEP.

Results revealed a remarkably low Michaelis-Menton constant (K_M) of 82 nM and a turnover of 1.65 molecules per second. We found that an N-terminally-truncated SpyCEP C-terminal construct containing just the catalytic dyad of H279 and S617 was capable of cleaving CXCL8 with a similar K_M of 55 nM, albeit with a reduced substrate turnover of 2.7 molecules per hour, representing a 2,200-fold reduction in activity. We conclude that the SpyCEP C-terminus plays a key role in high affinity substrate recognition and binding, but that the N-terminus is required for full catalytic activity.

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Key words: *Streptococcus pyogenes*, virulence factor, serine protease, SpyCEP

59 **Introduction**

60

61 Group A Streptococcus (GAS) or *Streptococcus pyogenes* is a leading human pathogen that manifests
62 clinically as a broad spectrum of diseases, ranging from less severe, usually self-limiting infections to
63 life-threatening invasive diseases such as necrotizing fasciitis and toxic shock syndrome. While much
64 of the global health burden of *S. pyogenes* can be attributed to auto-immune sequelae such as
65 rheumatic heart disease, invasive diseases contribute greatly to *S. pyogenes*-associated global
66 mortality. At least 663,000 new cases of invasive infection arise per year, accounting for
67 approximately 163,000 deaths [1] with mortality rates remaining high despite intervention; indeed,
68 20% of patients with invasive *S. pyogenes* disease die within 7 days of infection onset [2].

69

70 Several virulence factors contribute to pathogenesis in invasive *S. pyogenes* disease, chief among
71 which is *Streptococcus pyogenes* cell envelope protease (SpyCEP), an immune-modulatory cell wall-
72 associated protease. SpyCEP is responsible for the rapid and efficient cleavage of a distinct group of
73 CXC chemokines comprising CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, and CXCL8, both locally at
74 the site of an infection, and systemically [3-6]. The ELR^+ chemokines, named for their conserved N-
75 terminal glutamate-leucine-arginine motifs, specifically act upon neutrophils eliciting their
76 recruitment and activation. SpyCEP inactivates these chemokines by cleaving the chemokine C-
77 terminal α-helix, releasing a 13 amino acid peptide in the case of CXCL8 [3]. Although chemokine
78 specificity for binding neutrophil chemokine receptors, CXCR1 and CXCR2, is conferred to a large
79 extent by the chemokine N-terminal ELR motif [7], the chemokine C-terminus is necessary for
80 efficient receptor binding and activation [8], in addition to chemokine translocation from tissues to
81 endothelial lumen [7]. As such, SpyCEP cleavage of CXC chemokines results in a reduction of CXCR1
82 and CXCR2-mediated neutrophil chemotaxis and subsequent paucity of neutrophils at the site of *S.*
83 *pyogenes* infection [3]. SpyCEP has recently been shown to cleave the human anti-microbial peptide,
84 LL-37 in addition to chemokines [9]. Whilst cleavage does not affect the antimicrobial action of the
85 peptide, it was reported to reduce LL-37 specific neutrophil chemotaxis [9].

86 SpyCEP is expressed by *S. pyogenes* as a 1647 amino acid, 180 kDa subtilisin-like serine protease, the
87 crystal structure of which was recently solved to 2.8 Å resolution [10] and further refined to 2.2 Å
88 resolution [11]. It is a member of the S8 subtilase family, members of which are characterised by a
89 catalytic triad consisting of an aspartate, histidine, and serine residue, each surrounded by a region
90 of highly conserved amino acids [12]. SpyCEP is unique among streptococcal proteases in that,
91 during maturation, it is autocatalytically cleaved between residues Q244 and S245 into 2 distinct
92 polypeptides, a 30 kDa N-terminal polypeptide and a 150 kDa C-terminal polypeptide. The two
93 polypeptides harbour the separate residues required for the formation of the catalytic site [6]; the
94 N-terminal fragment contains the catalytic D151 residue, and the C-terminal fragment contains the
95 catalytic H279 and S617 residues. Upon cleavage, the two polypeptides re-associate non-covalently
96 to reconstitute the active enzyme [6]. The recent crystal structures have shed further light upon the
97 structure of SpyCEP, describing 9 separate domains, the first 5 of which contain the catalytic triad
98 necessary for enzymatic activity [10].
99
100 SpyCEP has been included as a target antigen in several recent *S. pyogenes* vaccine designs due to its
101 cell surface expression, highly conserved nature, and central role in *S. pyogenes* pathogenesis.
102 Immunisation with SpyCEP successfully elicits a SpyCEP-specific neutralising antibody response,
103 providing protection against systemic bacterial dissemination and reducing disease severity in *S.*
104 *pyogenes* intramuscular, skin infection models and non-human primate infection models [13-18].
105 Data that demonstrate vaccine dependence on enzyme inhibition highlight the importance of
106 understanding the enzymatic activity of SpyCEP and the potential to improve upon vaccine or
107 inhibitor design [13].
108
109 Despite this, little is known about the catalytic properties of the enzyme, except preliminary
110 structure-function studies [6, 10, 11, 19]. Published data largely focus on the impact of SpyCEP on
111 streptococcal pathogenesis and disease progression. In this study we generated multiple SpyCEP

112 constructs to confirm domains necessary for catalytic activity. We then used mass spectrometry to

113 determine the enzyme kinetics of two SpyCEP constructs for the natural substrate CXCL8.

114

115 **Methods**

116 **Cloning and purification of recombinant SpyCEP constructs in *Escherichia coli***

117 Codon-optimised SpyCEP gene constructs were expressed in *Escherichia coli* using synthetic gene
118 sequences (GenScript) from Spy_0416 in the SF370 *S. pyogenes* M1 genome [6, 20] representing full-
119 length enzyme, SpyCEP$^{34-1613}$, the N- terminal polypeptide, SpyCEP$^{34-244}$, and C-terminal polypeptide
120 SpyCEP$^{245-1613}$. Constructs were also generated with alanine substitutions to replace catalytic
121 residues in the N-terminal fragment (SpyCEP$^{34-244}$ D151A), and the C-terminal fragment (SpyCEP^{245-}
122 ^{1613} S617A). To enable downstream protein purification, both the full-length enzyme and C-terminal
123 polypeptides were expressed with a C-terminal 6-histidine tag and the N-terminal polypeptides were
124 expressed with an N-terminal FLAG tag and TEV linker.

125 All SpyCEP constructs were cloned into the vector pET-24B and expressed in BL21 (DE3) competent
126 *E. coli*, cultured in Terrific Broth medium supplemented with 50 μg/mL kanamycin at 37°C and
127 shaken at 200 rpm, for 3 hours. The cultures were induced with 0.5 mM Isopropyl β-D-1-
128 thiogalactopyranoside (IPTG), cooled to 15°C and shaken at 200 rpm for 16 hours before lysis by
129 sonication on ice. Full-length and C-terminal constructs were purified by Ni-IDA affinity
130 chromatography (GenScript) as per the manufacturer's instructions. N-terminal constructs were
131 purified by anti-flag M2 agarose resin chromatography (Sigma-Aldrich) as per manufacturer's
132 instruction, and further concentrated and purified by SP ion-exchange (Sigma-Aldrich) and Q FF ion-
133 exchange chromatography (GE Healthcare) as per manufacturer's instruction. All SpyCEP constructs
134 were subsequently concentrated and purified by size exclusion chromatography on a HiLoad
135 Superdex 200 or 75 prep grade column (GE Healthcare) dependent on molecular weight.

136 The resultant panel of recombinantly expressed SpyCEP constructs is shown in Table 1. All of the
137 resultant protein preparations were found to be >80% pure as determined following analysis by SDS-
138 PAGE. The re-association of the SpyCEP N and C-termini to create full-length constructs was carried
139 out by equimolar co-incubation at 37°C for 30 minutes in 40 mM Tris-HCl pH 7.5, 0.1 mM CHAPS, 1
140 mM DTT, 0.1% BSA, 75 mM NaCl.

SpyCEP construct	SpyCEP construct description
SpyCEP	Full-length, SpyCEP$^{34\text{-}1613}$-6His
SpyCEP$^{D151A,\ S617A}$	Full-length double mutant, SpyCEP$^{34\text{-}1613\ D151A,\ S617A}$-6His
N-terminal	N-terminal fragment, FLAG-TEV-SpyCEP$^{34\text{-}244}$
C-terminal	C-terminal fragment, SpyCEP$^{245\text{-}1613}$-6His
N-terminal^{D151A}	N-terminal mutant fragment, FLAG-TEV-SpyCEP$^{34\text{-}244\ D151A}$
C-terminal^{S617A}	C-terminal mutant fragment, SpyCEP$^{245\text{-}1613\ S617A}$-6His
s.pSpyCEP	SpyCEP from *S. pyogenes* supernatant, SpyCEP$^{34\text{-}1613}$-6His

Table 1. SpyCEP constructs used in this study. Constructs were expressed recombinantly in *E. coli* except for s.pSpyCEP that was expressed in, and purified from *S. pyogenes*.

Expression of histidine-tagged SpyCEP in *S. pyogenes*

To permit release of soluble His-tagged SpyCEP by *S. pyogenes*, the C-terminal anchor domain of SpyCEP was replaced by a stop codon in *S. pyogenes* strain H292, an *emm*81 strain that makes abundant SpyCEP [5]. A 549 base pair region immediately upstream of the SpyCEP cell wall anchor motif was amplified from *S. pyogenes* H292 genomic DNA using the primers 5'GGGAATTCTGTTGTCAGGTAACAGTCTTATCTTGCC -3' and 5'CCGAATTCACAACACTAGGCTTTTGCTGAGGTCGTTG -3'. *EcoRI* restriction sites were incorporated at the terminal ends of the primer sequences. The amplified DNA was cloned into the homologous recombination plasmid pUCMUT to produce the vector pUCMUT$_{CEP}$ which was transformed into One Shot TOP10 Chemically Competent *E. coli* (Thermo Fisher Scientific) according to the manufacturer's instructions. The nucleotide sequence of the SpyCEP C-terminus was subsequently further modified to encode a hexa-histidine tag by inverse PCR using pUCMUT$_{CEP}$ as the template and the primers 5'-TATCCTAGGTAGTGTTGTGAATTCGTAATCATGGTCATAG-3' and 5'-TATCCTAGGATGATGATGATGATGATGGGCTTTTGCTGAGGTCGTTG-3'. The amplification was performed using GoTaq Long PCR Master Mix (Promega) according to the manufacturer's

161 instructions. *AvrII* restriction sites, incorporated at the terminal ends of the primer sequences,
162 facilitated re-circulation of the amplified plasmid. The presence of a 6-His sequence in the modified
163 $pUCMUT_{CEP}$ construct (denoted $pUCMUT_{CEP\text{-}HIS}$) was confirmed by Sanger sequencing using the
164 pUCMUT sequencing primers 5'-GACAGCAACATCTTTGTGAAAGATGG-3' and 5'-
165 CATTAATGCAGCTGGCACGAC-3'. The $pUCMUT_{CEP\text{-}HIS}$ construct was introduced into H292 by
166 electroporation and crossed into the chromosome by homologous recombination as previously
167 described [21] to generate strain H1317. Secretion of 6-His-tagged SpyCEP into the culture
168 supernatant of H1317 was confirmed by western blotting (data not shown). To purify SpyCEP, *S.*
169 *pyogenes* H1317 was grown in Todd-Hewitt broth (Oxoid) overnight at 37°C with 5% $C0_2$ The culture
170 was pelleted at 2500 xg for 10 minutes and the supernatant sterilised using Amicon 0.22 µm filters,
171 then concentrated using 15 ml Amicon 100 kDa centrifuge filter columns and purified by nickel
172 column affinity chromatography (Novagen His-Bind Resin) as per the manufacturer's instructions.

173

174 **Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-page)**

175 To visualise chemokine cleavage, 50 pmol of recombinant human CXCL1 or CXCL8 (R&D Systems)
176 was incubated with C-terminal SpyCEP in a final volume of 20 µl, at a molar ratio of 1:5, 50, 500,
177 5000 in favour of chemokine for 2 hours at 37°C. C-terminal^{S617A} and N-terminal constructs$^{34\text{-}244}$ were
178 included as controls and assayed at the highest 1:5 molar ratio. Reactions were halted by the
179 addition of Dithiothreitol (DTT) at a final concentration of 100 mM, 4X Bolt LDS sample buffer and
180 heating to 70°C for 10 minutes. Samples were separated on pre-cast 4-12% MES buffered Bolt Bis-
181 Tris gradient gels (Invitrogen) by SDS-PAGE gel electrophoresis at 165 V for 35 minutes with SeeBlue
182 Plus 2 (Invitrogen) used for molecular weight ladder. Gels were stained with PageBlue protein
183 staining solution (Thermo Fisher Scientific) overnight and de-stained in deionised water.

184

185 **Multiplex fluorescent western blotting**

186 To visualise generation of both intact CXCL8 and the larger (N-terminal) CXCL8 cleavage product, a
187 rabbit antiserum was raised against the CXCL8 neo-epitope (anti-ENWVQ) that is exposed following
188 SpyCEP cleavage of CXCL8 [22]. SpyCEP constructs were incubated at 37 °C with 18.75 pmol of
189 recombinant human CXCL8 in a final volume of 20 µl, at a 1:50 molar ratio in favour of CXCL8.
190 Digests were halted and separated by SDS-PAGE gel electrophoresis as described above. Proteins
191 were transferred by iBlot2 (Thermo Fisher Scientific) onto 0.2 µm nitrocellulose membranes as per
192 manufacturer's instructions. Membranes were subsequently blocked for 1 hour at room
193 temperature in blocking buffer (PBS with 5% skimmed milk powder (Sigma-Aldrich) and 0.1%
194 Tween), then blotted overnight at 4 °C with 2 primary antibodies, 1 µg/ml mouse anti-human CXCL8
195 (R&D Systems) and 1:1000 rabbit anti-ENWVQ. Membranes were washed in wash buffer (PBS with
196 0.05% Tween) and incubated with 1:7500 goat anti-rabbit IgG A680nm and 1:7500 goat anti-mouse
197 IgG A790nm for 1 hour at room temperature before being visualised on LiCor Odyssey Fc
198 (Invitrogen).

199 SpyCEP activity against LL-37 was assessed by a 16 hour, 37 °C incubation of 111.1 pmol human LL-37
200 (R&D Systems) with SpyCEP constructs at a 1:10 molar ratio in favour of LL-37 in a final volume of 20
201 µl. Reactions were stopped, separated and blotted onto 0.2 µm nitrocellulose as above and
202 incubated overnight at 4°C in blocking buffer supplemented with 2 µg/ml polyclonal sheep IgG anti-
203 LL-37 (R&D Systems). Membranes were washed in wash buffer (PBS with 0.05% Tween) and
204 incubated in blocking solution with rabbit anti-sheep IgG (Abcam) at 1:40,000 dilution for 1 hour at
205 room temperature and visualised on LiCor Odyssey Fc (Invitrogen).

206 **Enzyme linked immunosorbent assay (ELISA) analysis of chemokine cleavage**

207 Catalytic activities of SpyCEP constructs were measured through detection of remaining intact CXCL8
208 or CXCL1 substrate following incubation with SpyCEP by ELISA (R&D Systems human CXCL8 and
209 CXCL1 DuoSet ELISA) as per manufacturer's instructions. For cleavage time courses of CXCL8 by C-

210 terminal SpyCEP, 5 pmol CXCL8 were incubated with C-terminal constructs at a range of enzyme:
211 chemokine molar ratios, 1:5 – 1:250, in a final volume of 100 µl at room temperature for 60 minutes.
212 Full-length SpyCEP and C-terminal ^{S617A} were included as controls at a molar ratio of 1:1000 and 1:5,
213 respectively. To compare SpyCEP cleavage rates of CXCL8 and CXCL1, 5 fmol or 10 fmol SpyCEP were
214 incubated with 2 pmol human CXCL8 and CXCL1 respectively (R&D Systems), in a final volume of 100
215 µl and incubated at room temperature for 30 minutes. All reactions were halted at defined
216 timepoints with the addition of concentration of Pefabloc (Sigma-Aldrich) to a final concentration of
217 2 mg/ml (8.34 mM).

218 Linear regression analyses of the initial five time points of CXCL1 and CXCL8 cleavage (0, 1, 2, 3 and 4
219 minutes) were utilised to determine the maximal rate of SpyCEP activity.

220 **Mass spectrometry analysis of CXCL8 cleavage**

221 Analysis of CXCL8 cleavage was assayed on a SCIEX API6500 triple quadrupole electrospray mass
222 spectrometer coupled to a high-throughput robotic sample preparation and injection system,
223 RapidFire200 (Agilent Technologies). CXCL8 substrate, the 13 amino acid CXCL8 SpyCEP cleavage
224 product RVVEKFLKRAENS, and a heavy atom substituted internal standard of CXCL8 SpyCEP cleavage
225 product RV [$U_{13}C_5$ $_{15}$N-VAL]-EKF-[U-$_{13}C_6$ $_{15}$N-Leu]- KRAENS) were monitored by mass spectrometry.
226 The mass spectrometer was operated in positive electrospray MRM mode, and transitions (Q1/Q3)
227 for each species were optimised to give m/z as follows: CXCL8, 1048.7/615.3, CXCL8 cleavage
228 product 526.2/211.1, internal standard 530.5/211.1. A dwell time of 50 ms was used for the MRM
229 transitions. The mass spectrometer was operated with a spray voltage of 5500 V and at a source
230 temperature of 650 °C.

231 To assay CXCL8 cleavage dynamically, chemokine and SpyCEP constructs were loaded into a 384 well
232 plate to the following final concentrations: 6.25-2000 nM chemokine, 250 pM SpyCEP or 40 nM C-
233 terminal SpyCEP in a 20 µl reaction volume. Reactions were carried out at 21°C and stopped at
234 desired time points, 0-240 minutes, by the addition of stop solution (1% formic acid) supplemented

235 with 1 µM heavy atom substituted CXCL8 internal standard and centrifuged 2000 xg for 10 minutes.
236 Assay plates were transferred onto the RapidFire200 integrated to the API6500 mass spectrometer.
237 Samples were aspirated under vacuum directly from 384-well assay plates for 0.6 s. The samples
238 were then loaded onto a C18 solid-phase extraction cartridge to remove non-volatile buffer salts,
239 using HPLC -grade water supplemented with 0.1% (v/v) formic acid at a flow rate of 1.5 mL/min for 4
240 s. The retained analytes were eluted to the mass spectrometer by washing the cartridge with
241 acetonitrile HPLC-grade water (8:2, v/v) with 0.1% (v/v) formic acid at 1.25 mL/min for 4 s. The
242 cartridge was re-equilibrated with HPLC-grade water supplemented with 0.1% (v/v) formic acid for
243 0.6 s at 1.5 mL/min.

244 Results were expressed as a ratio of CXCL8 cleavage product (526.2/211.1) intensity areas compared
245 with the internal standard (530.5/211.1) instensity areas. The results were interpolated from a
246 standard curve constructed using known amounts of cleaved CXCL8 over the range of 25 – 2000 nM
247 where there was a linear relationship (R^2=0.99) and with a coefficient of variation < 9% for all
248 concentrations. Where interpolated values were below 25 nM, the assay LOQ, values were assigned
249 an arbitrary value of LOQ/2, 12.5 nM.

250

251 **Kinetic analysis**

252 Linear regression analyses from five time points of full-length SpyCEP (1, 2, 3, 4, 5 minutes) and C-
253 terminal SpyCEP (15, 30, 45, 60, 75 minutes) CXCL8 reactions were plotted against substrate
254 concentration and kinetics derived from the Michaelis-Menton equation $Y = V_{max}*X/ (K_M + X)$ and the
255 K_{cat} equation $Y = ET*k_{cat}*X/(K_M + X)$ where Et = enzyme concentration as fitted by GraphPad Prism v
256 8.0.2.

257

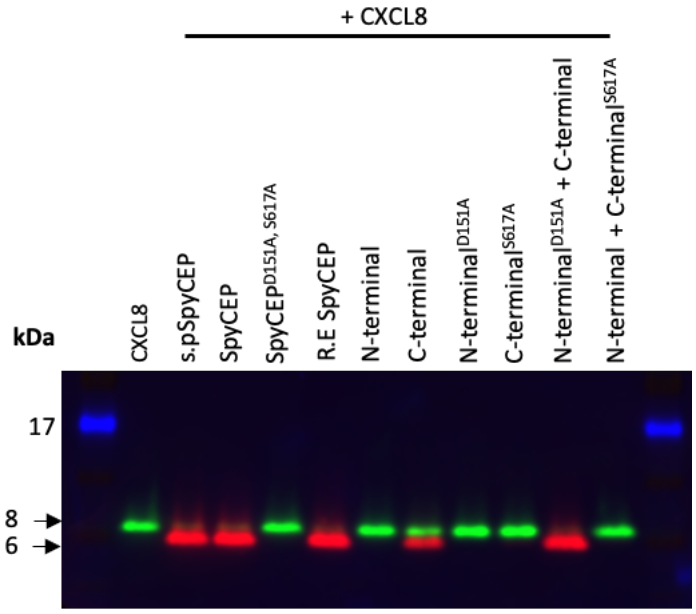
258 **Results**
259

260 ***Screening of CXCL8 cleavage activity using fluorescent western blotting***
261

262 To initially assess the activity of recombinant SpyCEP constructs we screened constructs for specific
263 CXCL8 cleavage activity using two-colour multiplex western blotting. Human CXCL8 was incubated
264 for 2 hours at 37 ^{o}C with the different SpyCEP constructs, and the reaction products were separated
265 by SDS-PAGE, then immunoblotted using separate antibodies that detect either intact or cleaved
266 CXCL8. Detection of full-length CXCL8 (8 kDa, green bands) or the CXCL8 neo epitope (ENWVQ),
267 exposed after SpyCEP cleavage, (6 kDa, red bands) was evident with this system (Figure 1).
268 Recombinant full-length SpyCEP expressed in *E. coli* successfully cleaved CXCL8 to completion, with
269 no difference observed between CXCL8 cleavage with *S. pyogenes* SpyCEP and *E. coli* SpyCEP (Figure
270 1). As expected, no CXCL8 cleavage was observed when using the catalytically inactive mutant,
271 SpyCEP$^{D151A, S617A}$. As has been previously reported [6], the SpyCEP N- and C-termini, when
272 independently expressed and purified, can be re-associated to form an active enzyme R.E SpyCEP,
273 which successfully cleaved CXCL8. The N-terminal fragment of SpyCEP alone could not cleave CXCL8.
274 Unexpectedly, however, the C-terminal fragment of SpyCEP was observed to cleave CXCL8, albeit
275 not to completion. This independent catalytic activity was negated by mutation of the catalytic S617
276 to alanine (C-terminal^{S617A} construct, Figure 1). Cleavage of CXCL8 by the SpyCEP C-terminal fragment
277 was enhanced when re-associated with the catalytically inert N-terminal mutant (N-terminal^{D151A}
278 construct); indeed, activity was equivalent to that observed with the re-associated enzyme under
279 these conditions, with cleavage of CXCL8 to near completion. However, the N-terminal^{D151A} construct
280 was unable to restore catalytic activity to the C-terminal^{S617A} construct when the two were
281 combined.

282 The results demonstrated for the first time that the SpyCEP C-terminal fragment alone is sufficient
283 for catalytic cleavage of CXCL8 in this assay system. The data also established that, although the
284 presence of the SpyCEP N-terminal fragment enhanced enzymatic activity, the N-terminal residue

285 D151 was dispensable for enzymatic activity. We further sought to examine whether the C-terminal
286 displayed activity against the newly described substrate, LL-37 [9]. Western blot analysis confirmed
287 cleavage of LL-37 by full-length recombinant SpyCEP, demonstrated by a reduction in band size,
288 however the C-terminal fragment was unable to cleave LL-37 despite a high molar ratio of enzyme to
289 LL-37 (1: 10) and a prolonged 16-hour incubation at 37 °C (Figure S1).



290

291 **Figure 1. Cleavage activity of recombinant SpyCEP constructs assayed by immunoblot.** Two colour
292 immunoblot showing cleavage of 18.75 pmol CXCL8 when incubated for 2 hours at 37 °C either alone
293 (2nd lane) or with a panel of SpyCEP constructs at a 1:50 molar ratio (SpyCEP: CXCL8). Green bands
294 represent intact CXCL8 (anti-CXCL8 antibody); red bands represent cleaved CXCL8 (anti- ENWVQ). A
295 17 kDa molecular weight marker is shown in blue, 8 kDa and 6 kDa molecular weights are highlighted
296 by arrows. Figure is representative of 2 independent immunoblots.

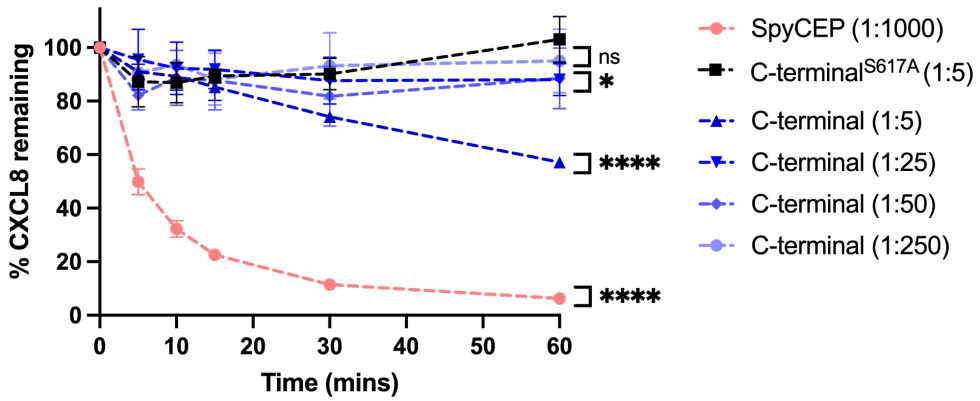
297

298

299 ***Differential cleavage of CXCL8 and CXCL1 by full-length and C-terminal SpyCEP***
300

301 To determine whether the catalytic activity of the C-terminal SpyCEP fragment was reproducible
302 over a shorter incubation period, we assessed CXCL8 cleavage by ELISA, where CXCL8 cleavage is
303 detected through a reduction in substrate concentration. SpyCEP C-terminal constructs were
304 incubated with CXCL8 at molar ratios ranging from 1:5 – 1:250 (enzyme: chemokine) over a 60-
305 minute time course at room temperature. Full-length recombinant SpyCEP and the inactive C-

306 terminal fragment, C-terminal^{S617A}, were included as controls at a molar ratio of 1:1000 and 1:5,
307 respectively. Under these conditions, near complete CXCL8 cleavage was observed for full-length
308 SpyCEP and a dose-dependent increase in catalytic activity was observed for the C-terminal fragment
309 (Figure 2). After 5 minutes incubation full-length SpyCEP cleaved over 50% of the starting CXCL8
310 input, with only 6.25% of CXCL8 remaining after 1 hour. At the highest concentration of C-terminal
311 SpyCEP tested, a 1:5 molar ratio, the C-terminal fragment alone cleaved 9% of the starting CXCL8 by
312 5 minutes, 25% by 30 minutes and 42% by 60 minutes. Indeed, at the lowest concentration tested, a
313 1:250 molar ratio, the C-terminal of SpyCEP cleaved 9% of CXCL8 input by 1 hour. As demonstrated
314 by immunofluorescent western blotting, the serine residue at position 617 was vital for SpyCEP
315 catalytic function, as no CXCL8 cleavage was observed using the C-terminal^{S617A} construct, even when
316 employed at the highest enzyme: chemokine ratio. After a 1-hour incubation, full-length SpyCEP and
317 C-terminal constructs (assayed using a molar enzyme: chemokine ratio of 1:5, 1:25 and 1:50) cleaved
318 significantly more CXCL8 compared to the C-terminal^{S617A} construct.



319

320 **Figure 2. Cleavage activity of SpyCEP and C-terminal SpyCEP$^{245-1613}$ constructs using CXCL8 ELISA.**
321 SpyCEP constructs were co-incubated with 5 pmol CXCL8. Graphs show residual CXCL8 after a 60-
322 minute room temperature incubation, using full-length SpyCEP at a 1:1000 ratio to CXCL8; the C-
323 terminal SpyCEP construct at a 1:5 – 1:250 molar ratio to CXCL8; and the C-terminal^{S617A} mutant at a
324 1:5 molar ratio to CXCL8. Reactions were halted at specified timepoints by the addition of Pefabloc
325 to a final concentration of 2 mg/ml. N=6 experimental replicates for each construct, data points
326 show means, error bars represent SD. ns $p > 0.05$, * $p \leq 0.05$, **** $p \leq 0.0001$, at 60 minutes as
327 determined by ordinary one-way ANOVA.

328

329 SDS-PAGE analysis of the catalytic activity of the C-terminal SpyCEP construct additionally showed
330 that C-terminal activity was not restricted to the CXCL8 substrate. Over 2 hours at 37 °C using a 1:5
331 molar ratio of enzyme: substrate, the SpyCEP C-terminal construct was capable of cleaving human
332 CXCL1 to near completion (Figure S2).

333 To further assess the activity of SpyCEP and to interrogate reaction rates against separate
334 chemokines, full-length SpyCEP was incubated with CXCL1 and CXCL8 and the remaining chemokine
335 levels determined by ELISA. Human CXCL1 or human CXCL8 were incubated with SpyCEP at room
336 temperature over a 30-minute time course, at 1:200 or 1:400 molar ratios respectively (enzyme:
337 chemokine). 5 fmol of SpyCEP rapidly and efficiently cleaved 2 pmol CXCL8, with ~ 15% of the
338 chemokine input remaining after 10 minutes of incubation (Figure 3A). This contrasted with CXCL1
339 cleavage, that required 10 fmol SpyCEP to cleave just 25% of the chemokine input over the same 10-
340 minute period. Indeed, by 30 minutes SpyCEP had yet to cleave half of the starting amount of CXCL1
341 (Figure 3B). Utilising a linear regression of the initial 5 timepoints, where SpyCEP activity is maximal,
342 we found that 5 fmol of SpyCEP was able to cleave 284 fmol of CXCL8 per minute, and 10 fmol of
343 SpyCEP was capable of cleaving 62 fmol of CXCL1 per minute. These data confirmed the activity of
344 recombinant SpyCEP and highlighted differential cleavage efficiency across the CXC substrate range
345 – a feature which has been previously recognised but not quantified [8].

346

347

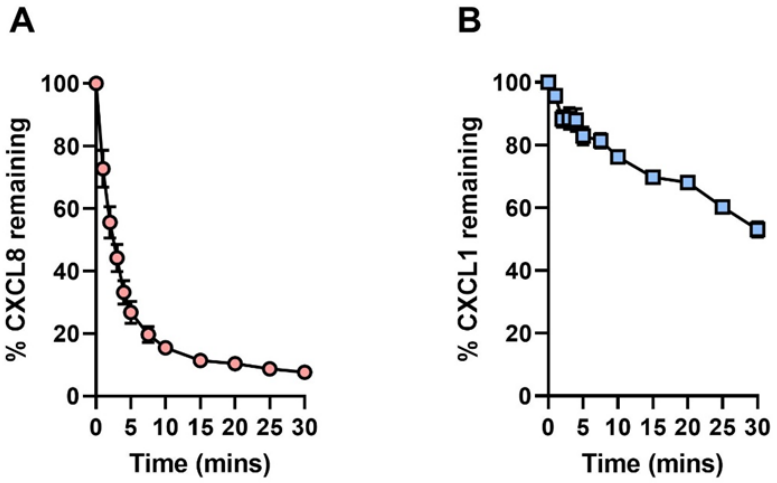


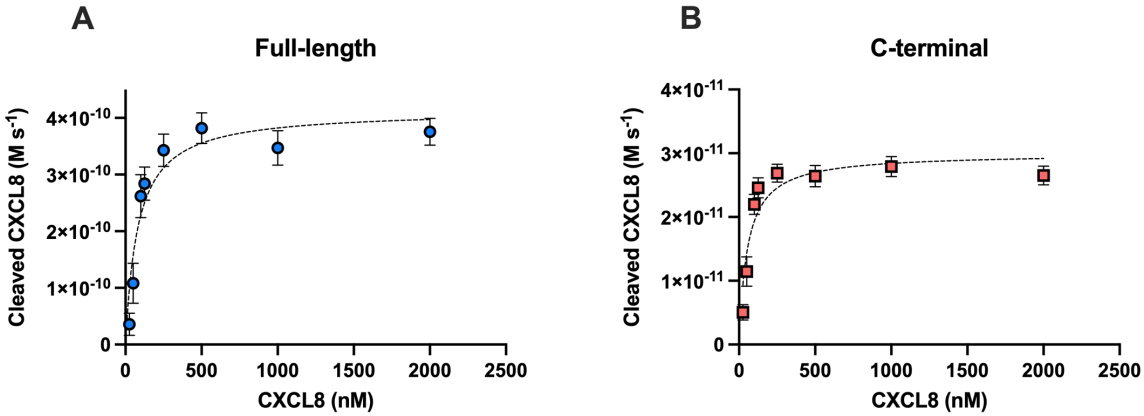
Figure 3. Cleavage of CXCL8 and CXCL1 by recombinant full-length SpyCEP. 30-minute, room temperature cleavage time course of: A. 2 pmol CXCL8 with 5 fmol SpyCEP and B. 2 pmol CXCL1 with 10 fmol SpyCEP. Cleavage reactions were halted at specified timepoints by the addition of Pefabloc to a final concentration of 2 mg/ml and the remaining chemokine measured by ELISA. N=6 experimental replicates for each data point, error bars represent SD of mean values.

Mass spectrometry-derived kinetics of active SpyCEP constructs

To directly compare the active full-length and C-terminal SpyCEP constructs and to understand relative catalytic efficiencies, a mass spectrometry approach to continuously assay the generation of the 13 amino acid peptide cleaved from the native substrate CXCL8, following incubation with enzyme, was employed. A range of CXCL8 concentrations (25-2000 nM) were incubated with a fixed concentration of enzyme, either 250 pM of full length SpyCEP, or 40 nM of the C-terminal SpyCEP$^{245-1613}$ and the production of the 13 amino acid peptide was monitored over time. 250 pM full-length SpyCEP cleaved CXCL8 to near completion over 30 minutes when substrate concentrations were less than 250 nM; incomplete CXCL8 cleavage was observed when substrate concentration was in excess of 250 nM (Figure S3A). In contrast, 40 nM of the C-terminal SpyCEP$^{245-1613}$ cleaved CXCL8 to near completion over 4 hours when the CXCL8 concentration was 250 nM or less; incomplete CXCL8 cleavage was again observed when substrate concentrations were over 250 nM (Figure S3B). Under these conditions the C-terminal SpyCEP fragment maintained measurable catalytic activity

378 throughout, though with reduced efficacy compared to the full-length construct. A 160-fold increase
379 in enzyme concentration and additional 3.5 hours reaction time were required to cleave a
380 comparable amount of CXCL8.

381 Linear regression analyses of 5 time points, where the rate of cleaved CXCL8 production was linear,
382 were used to derive Michaelis-Menton plots (Figure 4) and K_M and K_{cat} values for each construct
383 (Table 2).



384

385 **Figure 4. Kinetic activity of full-length and C-terminal SpyCEP construct cleavage of CXCL8.** Data
386 points represent the mean change in cleaved CXCL8 production over time (M s^{-1}) of, **A.** Full-length
387 SpyCEP and **B.** C-terminal SpyCEP. Error bars represent the standard error of the mean of 5
388 reactions.

389

Construct	**K_{cat} ± SEM (s^{-1})**	**K_M ± SEM (nM)**	**V_{max} ± SEM (M s^{-1})**	**K_{cat}/K_M (M^{-1} s^{-1})**
Full-length	1.65 ± 0.13	82 ± 26	4.14 x10^{-10} ± 3.35 x10^{-11}	2.02 x10^{7}
C-terminal	0.00075 ± 0.00005	55 ± 17	3.00 x10^{-11} ± 2.17 x10^{-12}	1.36 x10^{4}

390

391 **Table 2. Kinetic parameters of full-length and C-terminal SpyCEP construct activity in cleavage of**
392 **CXCL8**

393 K_{cat}, K_M and V_{max} ± SEM and K_{cat} / K_M for full-length SpyCEP and C-terminal SpyCEP derived from
394 Michaelis-Menton graphs $Y = V_{max}*X/ (K_M + X)$ and K_{cat} equation $Y = ET*k_{cat}*X/(K_M + X)$ where Et =
395 enzyme concentration, 2.5 x10^{-10} M for full-length SpyCEP and 4 x10^{-8} M for C-terminal SpyCEP.

396

397 K_M values for full-length SpyCEP and the C-terminal fragment were 82 nM and 55 nM respectively,
398 suggesting nanomolar affinity of the SpyCEP constructs for CXCL8. The similarity in the K_M values
399 between the C- terminal SpyCEP fragment and the full-length enzyme point to a similar ability to
400 bind substrate. The K_{cat} values (indicative of the number of molecules of cleaved CXCL8 produced per
401 second) however, differed substantially. The full-length enzyme cleaved 1.65 molecules of CXCL8 per
402 second compared to the C-terminal construct which cleaved 2.7 molecules per hour, a 2,200-fold
403 reduction in activity.

404

405 **Discussion**
406

407 SpyCEP is a serine protease and a leading virulence factor of *S. pyogenes,* with a narrow range of
408 substrate specificity, restricted to the family of ELR^{+} CXC chemokines which modulate neutrophil
409 mediated immune responses [3, 4, 8] and LL-37 [9]. Autocatalytic processing of SpyCEP results in the
410 generation of two individual fragments that re-assemble to form an active enzyme [6]. Here, we
411 describe the enzyme kinetics of full-length SpyCEP and report the K_M of the enzyme for its natural
412 substrate to be remarkably low, just 81.76 nM, consistent with high efficiency. Furthermore, we
413 demonstrate that the C-terminal SpyCEP fragment can catalyse the cleavage of CXCL1 and CXCL8
414 independent of the N-terminal fragment. Indeed, when K_M values were compared, they were found
415 to be similar, suggesting that substrate binding may be confined to the C-terminal domain of
416 SpyCEP. The enzymatic activity of the C-terminal SpyCEP fragment was, however, markedly reduced
417 compared to full-length SpyCEP. The N-terminal and N-terminal^{D151A} constructs were equally able to
418 restore full catalytic activity of the C-terminal SpyCEP fragment. Collectively, this suggests that
419 although the aspartate 151 of the N-terminal fragment may be dispensable for catalysis, the domain
420 itself is important for optimal enzyme activity.

421 Serine proteases are ubiquitous and comprise up to one third of all proteolytic enzymes currently
422 described. They are currently categorised by the MEROPS database [23] into 13 distinct clans, being
423 differentiated into groups of proteins based on their evolution from the same common ancestor,
424 with SpyCEP belonging to the S8 family of the SB clan. S8 serine proteases are typified by a classical
425 catalytic triad composed of serine, histidine and aspartic acid residues that together contribute to
426 the hydrolysis of a peptide bond within the substrate. It is recognised that a number of serine
427 protease clans employ a variation on the S8 catalytic triad, utilising instead a triad of serine,
428 histidine, and glutamic acid, or serine, glutamic acid, and aspartic acid residues. Other clans utilise
429 catalytic dyads of lysine and histidine or histidine and serine for proteolytic activity. Our data suggest
430 that SpyCEP activity can reside in a catalytic dyad of histidine and serine, albeit at a greatly reduced

431 efficacy. It is likely this large gulf in efficiency explains the failure of previous studies to detect
432 catalytic activity within the isolated C-terminal SpyCEP fragment [6].

433 Within serine proteases there are additional features, beyond the catalytic triad residues, which can
434 contribute to activity. The oxyanion hole for example, a pocket in the active site composed of
435 backbone amide NH groups, may provide substrate stabilisation and help drive catalysis [24].
436 Additional residues located in close proximity to the catalytic pocket can also mitigate a loss of
437 activity resulting from a missing residue, and water also has the potential to moonlight as a missing
438 functional group [24]. These 'stand ins' can provide a possible substitute machinery to help drive
439 catalytic function. Indeed, some studies have shown that, even with all three catalytic triad residues
440 removed, serine proteases are still capable of catalysis at rates 1000-fold greater than the
441 background rate of hydrolysis [24-26].

442 Mass spectrometry-based kinetics showed that full-length active SpyCEP has a K_M of 82 nM and K_{cat}
443 of 1.65 molecules per second; values which are in agreement with our initial ELISA-based kinetic
444 assessment [11]. In contrast, the C-terminal fragment of SpyCEP has a K_M of 55 nM and K_{cat} of
445 0.00075 molecules per second. These constructs both demonstrated low, nanomolar K_M values,
446 suggesting a high binding efficiency of SpyCEP for its natural substrate CXCL8, likely conferred by the
447 C-terminal domain. This is in keeping with the fact that low nanomolar concentrations of CXCL8 are
448 optimal for neutrophil recruitment [8]. Although K_M values are often reported in the μM – mM
449 range, nanomolar K_M values are not without precedent for other serine proteases; human kallikrein
450 6 has a reported K_M of 300 nM and Factor Xa, a constituent of the prothrombinase complex, has a K_M
451 of 150 nM for prothrombin [27, 28]. Enzyme specificity, a constant which measures the cleavage
452 efficiency of enzymes, (K_{cat}/K_M), for full-length SpyCEP was estimated to be 2.02 $x10^7$ M^{-1} s^{-1}, a
453 value in the order of magnitude typical for serine proteases [24]. The specificity constant of the C-
454 terminal fragment was ~1500-fold less, 1.36 $x10^4$ M^{-1} s^{-1}, and K_{cat} 2200 fold less, a reduction that is in
455 line with previously reported aspartic acid mutants from a systematic mutational study of the

456 *Bacillus amyloliquefaciens* subtilisin catalytic triad [26]. The development of an MS-based assay of
457 SpyCEP activity, that is not dependent on western blotting or ELISA, provides potential for future
458 high-throughput analysis of SpyCEP activity and detection of SpyCEP inhibitors.

459 The kinetic assays attributed a marked increase in CXCL8 turnover to the additional presence of the
460 N-terminal fragment. Although we did not specifically evaluate the role of the aspartate residue at
461 position 151 on the kinetics of SpyCEP activity, this residue did not contribute appreciably to
462 cleavage of CXCL8 when evaluated using immunoblotting. This raises a question as to whether the N-
463 terminal fragment confers some additional structural contribution to enzyme activity. Our data
464 strongly suggest that substrate binding is likely to be attributed to the C-terminal fragment, a finding
465 consistent with related cell envelope proteinases of *Lactococci* [12] and the closely related
466 streptococcal protein, C5a peptidase [29].

467 The implications of our findings relating to activity of the C-terminal SpyCEP fragment in *S. pyogenes*
468 pathogenesis are currently unclear; without the N-terminus, the enzymatic activity detected may be
469 too low to be of consequence for chemokine cleavage *in vivo,* and in nature both N- and C-terminal
470 fragments are likely to co-exist. SpyCEP has been a focus of *S. pyogenes* vaccine development in
471 recent years [30], used either in isolation or combination with other antigenic targets [11, 14, 16, 17,
472 31]. Vaccine-induced SpyCEP specific antibodies appear not to act through traditional opsonic means
473 [13] and so may act through inhibition of SpyCEP activity. Many vaccine preparations evaluated have
474 been based on 'CEP5'; a polypeptide spanning residues 35 – 587 of SpyCEP which contains the N-
475 terminal fragment and only part of the C terminal fragment [18]. Our findings relating to enzyme
476 function suggest that antibodies targeting the C-terminal fragment of SpyCEP are more likely to
477 provide greater neutralizing activity, and potentially improve vaccine efficacy.

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Data availability

No new datasets were generated in this study other than those reported in the manuscript.

Author contributions

MP, CH, AF, DM, JP and SS designed the study. MP, CH, MR, RJE, RAL and JB collected the data. MP and CH analysed the data. MP, CH, AF, DM, JP and SS interpreted the data. MP collected and prepared the figures. MP, MR, JP and SS drafted the manuscript. MP, CH, AF, MR, RJE, RAL, DM, JP, SS, revised the manuscript content.

Conflict of interest

Co-authors Carl Haslam, Andrew Fosberry, Emma J Jones and Danuta Mossakowska were employed by GlaxoSmithKline R&D at the time this work was conducted. All other authors declare no conflict of interest.

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