

Supplementary Information

Impact of double covalent binding of BV in NIR FPs on their spectral and physicochemical properties

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Table S1. Molecular weight of peptides obtained by complete cleavage of iRFP670 in the holoform with chymotrypsin (cleavage after residues F, Y, and W) and trypsin (cleavage after residues K and R).

	peptides, kDa
chymotrypsin	7.13 ^{1,*} (<i>peptide A-BV-peptide B</i>); 5.04 ² (<i>peptide A</i>); 4.34; 3.48; 2.69; 2.26; 2.09 ^{3,*} (<i>BV-peptide B</i>); 2.08; 1.80; 1.74; 1.58; 1.46; 1.12; 1.02; 0.96; 0.82; 0.79; 0.62; 0.6; 0.35; 0.32; 0.26; 0.17
trypsin	3.31; 2.79 (<i>peptide A-BV-peptide B</i>); 2.49; 2.40; 2.38; 2.33; 2.12; 1.99; 1.88 (<i>BV-peptide B</i>); 1.40; 1.25; 1.18; 1.03; 0.99; 0.96; 0.91 (<i>peptide A</i>); 0.91; 0.77; 0.68; 0.66; 0.65; 0.62; 0.60; 0.52; 0.50; 0.47; 0.39; 0.38; 0.37; 0.25; 0.17; 0.15

¹ BV is covalently bound to two cysteines: Cys^{PAS} of peptide A and Cys^{GAF} of peptide B;

² peptide A contains Cys^{PAS};

³ peptide B contains Cys^{GAF} to which BV is covalently attached;

* the corresponding peptides contain a covalently attached BV and can be identified by Zn-induced fluorescence

Table S2. Molecular weight of peptides obtained by cleavage of iRFP670 in the apoform with chymotrypsin (cleavage after residues F, Y, and W).

	complete cleavage	partial cleavage
peptides, kDa	5.04 ¹ (<i>peptide A</i>); 4.34; 3.48; 2.69; 2.26; 2.08; 1.80; 1.74; 1.58; 1.51 ² (<i>peptide B</i>); 1.46; 1.12; 1.02; 0.96; 0.82; 0.79; 0.62; 0.6; 0.35; 0.32; 0.26; 0.17	6.41 or 4.97 ³

¹ peptide A contains Cys^{PAS};

² peptide B contains Cys^{GAF};

³ peptide B, formed if the polypeptide chain is not cleaved at sites Y213, F247. Numbering corresponds to sequence alignment of iRFP670 relative to *RpBphP2* (4E04 file in PDB [1]).

RpBphP2 (4E04)	1 MTEGSVARQP 10	20 DLSTCDEPI	30 HIPGAIQPHG	40 LLLALAADMT	50 IV-AGSDNLP	60 ELTGIAIGAL
iRFP670	MARK-----V	DLTSCDREPI	HIPGSIQPCG	CLLACDAQAV	RITHITENAG	AEFGRETPRV
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	PAS					
RpBphP2 (4E04)	70 IGRSAADVFD	80 SETHNRLTIA	90 LAEPGAAVGA	100 PIAVGFTMPD	110 GERA FN GSWH	120 RHDQLVFLEL
iRFP670	GELLADYFGE	TEAHALFNAL	AQSSDPKIPA	LIIGNRDGLT	GRITD-ISLH	RHDGTSIIEE
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	PAS					
RpBphP2 (4E04)	130 EPPQRDVRY P	140 QAFFRSVRSA	150 IRR LQA AETL	160 ESACAAA AQE	170 VREITGFDRV	180 MIYRFASDFS
iRFP670	EPAAAEQADN	PL--ELTRQI	IARTKELKSL	EEMAAIVPRY	LQAMLGVHRV	MLVFEADDGS
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	PAS	GAF				
RpBphP2 (4E04)	190 GEVIAEDRCA	200 EVESYLGLHF	210 PASDIPAQAR	220 RLYTINPVRI	230 IPDIN YRPVP	240 VTPDLNPR TG
iRFP670	GMVIGEA KFS	DLESFLGQHF	PASLVPQQA R	LLKLN A I R V	VSDSFGISSR	IVPEHDAS-G
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	GAF					
RpBphP2 (4E04)	250 RPIDLSFAIL	260 RSVSPVHLEY	270 MRNIGMHGTM	280 SISILRGERL	290 WGLIACHHRK	300 PNYVDLEVRQ
iRFP670	AALDLSFAHL	RSISPC HLE F	LRNMGVSASM	SLSIIDGTL	WGLIICHHIE	PAVPM AQR V
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	GAF					
RpBphP2 (4E04)	310 ACELV AQVLA	320 WQIGVMEEQA				
iRFP670	AAEMFADQLS	LHETAAHHQF				
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	GAF					

Figure S1. Alignment of amino acid sequences of iRFP670 with their wild-type templates *RpBphP2*-PAS-GAF. The highlighted in magenta are the conservative Cys residue in the PAS domain (Cys15) and the unique Cys residue in the GAF domain of iRFP670 (Cys256). The amino acids Phe, Tyr and Trp, preceding the chymotrypsin-cleavable amide bond, and Lys and Arg, preceding the trypsin-cleavable amide bond, are highlighted in red and green, correspondingly.

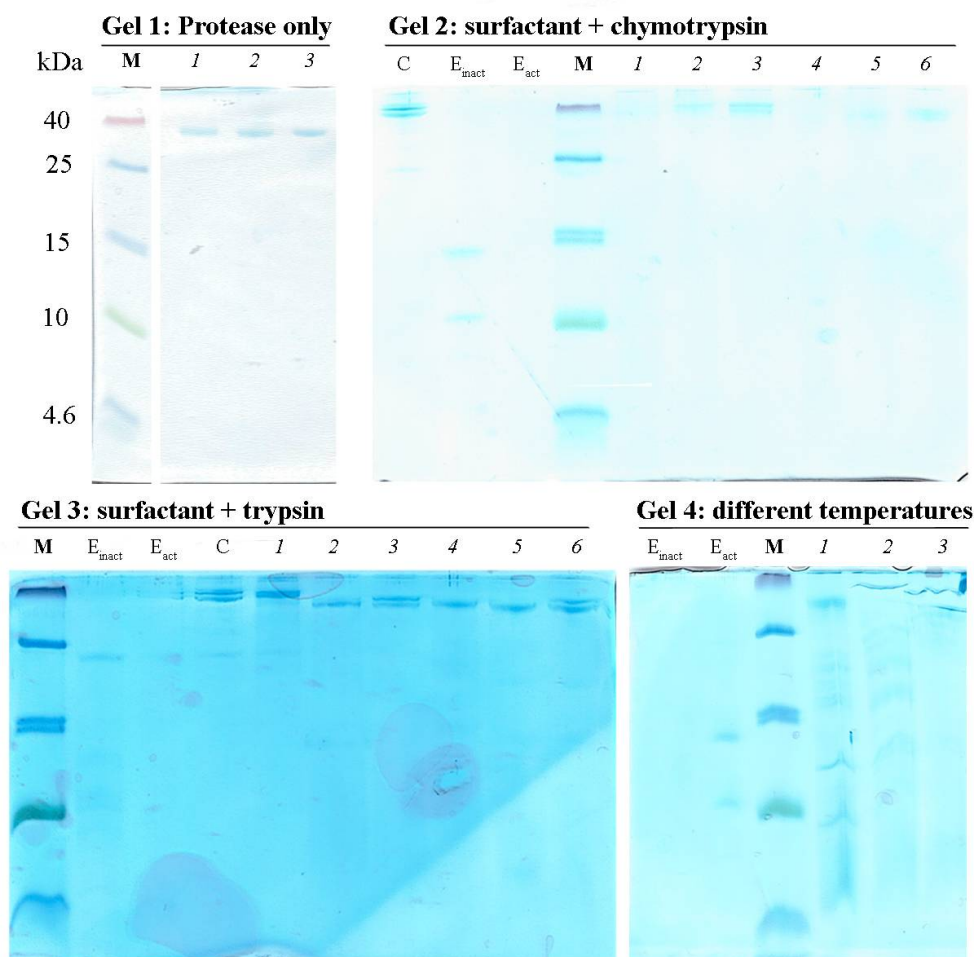


Figure S2. Cleavage of iRFP670 in the holoform under different conditions.

Tricine-SDS PAGE of protein samples followed by staining with Coomassie blue (CB). The following conditions were checked:

Gel 1, lanes 1 and 2 – protein cleavage with chymotrypsin at 50 °C without and with preliminary incubation of samples in the presence of 5 M urea; lane 3 – protein cleavage by trypsin at 37 °C. Gel 2, lanes 1, 2, and 3 – protein cleavage with chymotrypsin at 50 °C with preliminary incubation of samples in the presence of 2 M urea and ProteaseMAX™ Surfactant at concentrations of 0.2, 0.1, and 0.025%; lanes 4, 5, and 6 – protein cleavage with chymotrypsin at 50 °C with preliminary incubation of samples in the presence of ProteaseMAX™ Surfactant at concentrations of 0.2, 0.1, and 0.025%.

Gel 3, lanes 1, 2, and 3 – protein cleavage with trypsin at 37 °C with preliminary incubation of samples in the presence of 2 M urea and ProteaseMAX™ Surfactant at concentrations of 0.2, 0.1, and 0.025%; lanes 4, 5, and 6 – protein cleavage with trypsin at 37 °C with preliminary incubation of samples in the presence of ProteaseMAX™ Surfactant at concentrations of 0.2, 0.1, and 0.025%.

Gel 4, lanes 1, 2, and 3 – protein cleavage with chymotrypsin at 4, 37, and 50 °C with preliminary incubation of samples in the presence of 0.2% surfactant.

M – marker peptides, C – control samples of the original protein. The activity of proteases is confirmed by their self-cleavage at the optimum temperature. (E_{act}). Solutions of proteases whose activity is blocked (by addition of a PMSF inhibitor at a concentration of 1 mM at 4 °C) contain only peptides with a molecular weight of about 14 and 10 kDa for chymotrypsin and 23.3 kDa for trypsin, which corresponds to intact enzyme subunits (E_{inact}).

References

- [1] D. Bellini, M.Z. Papiz, *Acta Crystallogr D Biol Crystallogr*, Dimerization properties of the RpBphP2 chromophore-binding domain crystallized by homologue-directed mutagenesis, 68 (2012) 1058-1066.