Supporting Online Material

Rational design of a novel propeptide for improving active production of *Streptomyces griseus* trypsin in *Pichia pastoris*

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Running title: Rational design of a propeptide for trypsin production

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30 **TABLE S1** Strains and plasmids used in this study

Strains and plasmids	Genotype and characteristics	Reference
Strains		
S. griseus ATCC 10137^{TM}	*Streptomyces griseus* subsp. *griseus*	(1)
P. pastoris GS115	*his4* Mut^{+} His^{-} (*aox1*$^{+}$, *aox2*$^{+}$),	(2)
E. coli JM109	F′*traD36 proA*$^{+}$*B*$^{+}$ *lacIq* Δ*(lacZ)M15/*Δ*(lac-proAB)*	Lab stock
Exmt	*P. pastoris* GS115 harboring pPIC9k-Exmt	This study
ExAPNPmt	*P. pastoris* GS115 harboring pPIC9k-ExAPNPmt	This study
ExPNPmt	*P. pastoris* GS115 harboring pPIC9k-ExPNPmt	This study
ExNPmt	*P. pastoris* GS115 harboring pPIC9k-ExNPmt	This study
ExPmt	*P. pastoris* GS115 harboring pPIC9k-ExPmt	This study
ExYmt	*P. pastoris* GS115 harboring pPIC9k-ExYmt	This study
APNPmt	*P. pastoris* GS115 harboring pPIC9k-APNPmt	This study
Pmt	*P. pastoris* GS115 harboring pPIC9k-Pmt	This study
mt	*P. pastoris* GS115 harboring pPIC9k-mt	This study
RIRImt	*P. pastoris* GS115 harboring pPIC9k-RIRImt	This study
YVERmt	*P. pastoris* GS115 harboring pPIC9k-YVERmt	This study
YVEImt	*P. pastoris* GS115 harboring pPIC9k-YVEImt	This study
RVEFmt	*P. pastoris* GS115 harboring pPIC9k-RVEFmt	This study
IVEFmt	*P. pastoris* GS115 harboring pPIC9k-IVEFmt	This study
Plasmids		
pPIC9k	*HIS4 Amp*^{r} or *Kan*^{r}	(2)
pPIC9k-Exmt	pPIC9k inserted of fragment Exmt	This study
pPIC9k-ExAPNPmt	pPIC9k inserted of fragment ExAPNPmt	This study
pPIC9k-ExPNPmt	pPIC9k inserted of fragment ExPNPmt	This study
pPIC9k-ExNPmt	pPIC9k inserted of fragment ExNPmt	This study
pPIC9k-ExPmt	pPIC9k inserted of fragment ExPmt	This study
pPIC9k-ExYmt	pPIC9k inserted of fragment ExYmt	This study
pPIC9k-APNPmt	pPIC9k inserted of fragment APNPmt	This study
pPIC9k-Pmt	pPIC9k inserted of fragment Pmt	This study
pPIC9k-mt	pPIC9k inserted of fragment mt	This study
pPIC9k-RIRImt	pPIC9k inserted of fragment RIRImt	This study
pPIC9k-YVERmt	pPIC9k inserted of fragment YVERmt	This study
pPIC9k-YVEImt	pPIC9k inserted of fragment YVEImt	This study
pPIC9k-RVEFmt	pPIC9k inserted of fragment RVEFmt	This study
pPIC9k-IVEFmt	pPIC9k inserted of fragment IVEFmt	This study

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TABLE S2 Primers used in this study

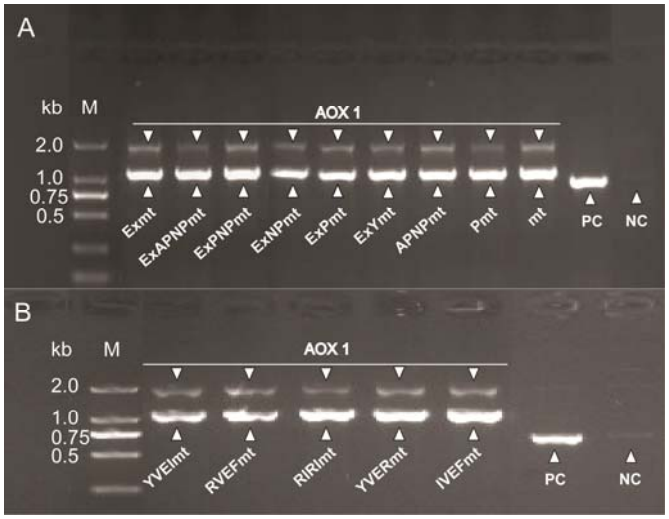
Primers	Sequence (5′→3′)
Exmt-5′	CCGGAATTCGTCGTCGGCGGAACCC
ExAPNPmt-5′	CCGGAATTCGCTCCAAACCCAGTCGTCGGCGGAACCC
ExPNPmt-5′	CCGGAATTCCCAAACCCAGTCGTCGGCGGAACCC
ExNPmt-5′	CCGGAATTCAACCCAGTCGTCGGCGGAACCC
ExPmt-5′	CCGGAATTCCCAGTCGTCGGCGGAACCC
ExYmt-5′	CCGGAATTCTACGTCGTCGGCGGAACCC
αF-5′	CGGGATCC**AAACG**ATGAGATTTCCTTCAATTTTTACTGC
APNPmt-5′	AGTTTCAGCCTCTCTTTTCTCGGCTCCAAACCCAGTCGTCGGCGGAACCC
αF-APNPmt-3′	GGGTTCCGCCGACGACTGGGTTTGGAGCCGAGAAAAGAGAGGCTGAAGCT
Pmt-5′	AGTTTCAGCCTCTCTTTTCTCGCCAGTCGTCGGCGGAACCC
αF-Pmt-3′	GGGTTCCGCCGACGACTGGCGAGAAAAGAGAGGCTGAAGCT
mt-5′	AGTTTCAGCCTCTCTTTTCTCG GTCGTCGGCGGAACCC
αF-mt-3′	GGGTTCCGCCGACGACCGAGAAAAGAGAGGCTGAAGCT
RIRImt-5′	AGTTTCAGCCTCTCTTTTCTCGAGAATTAGAATTGTCGTCGGCGGAACCC
αF-RIRImt-3′	GGGTTCCGCCGACGACAATTCTAATTCTCGAGAAAAGAGAGGCTGAAGCT
YVERmt-5′	AGTTTCAGCCTCTCTTTTCTCGTACGTTGAAAGAGTCGTCGGCGGAACCC
αF-YVERmt-3′	GGGTTCCGCCGACGACTCTTTCAACGTACGAGAAAAGAGAGGCTGAAGCT
YVEImt-5′	AGTTTCAGCCTCTCTTTTCTCGTACGTTGAAATTGTCGTCGGCGGAACCC
αF-YVEImt-3′	GGGTTCCGCCGACGACAATTTCAACGTACGAGAAAAGAGAGGCTGAAGCT
RVEFmt-5′	AGTTTCAGCCTCTCTTTTCTCGAGAGTTGAATTTGTCGTCGGCGGAACCC
αF-RVEFmt-3′	GGGTTCCGCCGACGACAAATTCAACTCTCGAGAAAAGAGAGGCTGAAGCT
IVEFmt-5′	AGTTTCAGCCTCTCTTTTCTCGATTGTTGAATTTGTCGTCGGCGGAACCC
αF-IVEFmt-3′	GGGTTCCGCCGACGACAAATTCAACAATCGAGAAAAGAGAGGCTGAAGCT
* mt-3′	ATAAGAATGCGGCCGCTCAGAGCGTGCGGGCGG
AOX primer-5′	GACTGGTTCCAATTGACAAGC
AOX primer-3′	TCCTACAGTCTTACGGTAAACGG

* mt-3′ was used as the downstream primer for all the recombinant trypsins or trypsinogens; The base underlined means the restriction enzyme sites; The base in bold means the Kozark sequence.

TABLE S3 Calculated parameters of the simulated structure of the trypsin mutants

Mutants	Residues	H-bonds	Ion pairs	π-interactions	Secondary structure %			
					α-helix	β-sheet	turn	coil
SGT	223	620	11	1	10.3%	39.9%	21.1%	28.7%
Exmt	227	312	8	5	8.0%	25.7%	17.3%	49.1%
ExAPNPmt	231	306	9	4	5.2%	22.2%	17.0%	55.7%
ExPNPmt	230	147	0	0	7.0%	26.1%	14.3%	52.6%
ExNPmt	229	123	1	4	7.0%	25.8%	11.8%	55.5%
ExPmt	228	126	0	1	5.7%	25.0%	15.4%	53.9%
ExYmt	228	164	3	4	8.8%	25.4%	17.5%	48.2%
APNPmt	227	113	2	0	8.4%	26.0%	10.6%	55.1%
Pmt	224	120	3	0	4.9%	29.0%	9.8%	56.3%
RIRImt	227	163	5	5	8.8%	27.8%	17.6%	45.8%
YVERmt	227	165	8	4	8.8%	26.4%	17.6%	47.1%
YVEImt	227	159	6	6	8.8%	27.3%	17.6%	46.3%
RVEFmt	227	174	6	4	8.8%	26.4%	17.6%	47.1%
IVEFmt	227	176	7	5	8.8%	25.6%	17.6%	48.0%

FIG S1 PCR confirmation of the integrated transformants with AOX primer-5′ and AOX primer-3′. (A) Confirmation of the trypsin mutants with the native propeptide and its stepwise deletion mutants. (B) Confirmation of the trypsin mutants with designed propeptides.



Construction of the recombinant vectors

Streptomyces griseus ATCC 10137 was purchased from the American Type Culture Collection and used for amplification of the trypsin gene. *Pichia pastoris* GS115 and the chromosome-integrated vector pPIC9k both were purchased commercially (Invitrogen, San Diego, CA, USA). The liberated inserts and digested expression vectors were electrophoresed and recovered using Agarose Gel DNA Purification Kit Ver.2.0 (TaKaRa, Dalian, China). The recombinant vectors were confirmed by sequencing using primers AOX primer-5′ and AOX primer-3′ (Table S2).

Oligonucleotides Exmt-5′/mt-3′, ExAPNPmt-5′/mt-3′, ExPNPmt-5′/mt-3′, ExNPmt-5′/mt-3′, ExPmt-5′/mt-3′ and ExYmt-5′/mt-3′ (Table S2) were used for amplification and construction of recombinants Exmt, ExAPNPmt, ExPNPmt, ExNPmt, ExPmt and ExYmt (Table S1), respectively. After digesting by *Eco*RI and *Not*I, the fragments were subcloned into pPIC9k (Fig. 1B). For construction of the recombinants APNPmt, Pmt, mt, RIRImt, YVERmt, YVEImt, RVEFmt and IVEFmt (Table S1), the α-factor signal peptide was welded by the fusion PCR method. After successful assembly, the recombinant fragments were digested with *BamH*I and *Not*I and subcloned into pPIC9k to generate the correspoding plasmids (Table S1).

Purification and SDS-PAGE analysis

All the trypsin mutants were purified by affinity chromatography. After centrifugation at 8,000 rpm for 10 min, the culture supernatant was concentrated and was further precipitated with 25-55% ammonium sulfate. The sediment was resuspended in 5 ml of buffer A (10 mM Tris-HCl, pH 8.0, 10% glycerol, and 1 mM EDTA) and dialyzed overnight against 100 ml buffer A. The sample was loaded onto a Hitrap benzamidine FF column (Φ1.6×2.5 cm, GE Healthcare, catalog number 17-5144-01) previously equilibrated with buffer A, and the column was washed with buffer B (10 mM NaOAc, pH 5.0, 10% glycerol, 1 mM EDTA, and 0.5M NaCl). After that, 10 μl ultrafiltrated purified enzymes supernatant were loaded onto the gel by SDS-PAGE analysis and the single bands showed in the pictures are the recombinant trypsins.

Determination of trypsin activity

Trypsin activity was measured spectrophometrically as the release of *p*-nitroaniline by enzymatic hydrolysis of the artifical substrate N_{α}-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA). Briefly, samples (100 μl) were mixed with 800 μl of assay buffer (50 mM Tris-HCl, pH 8.0; 0.02

M $CaCl_2$ at 37°C) and 100 µl 0.1 M BAPNA. The change in absorbance at 410 nm was recorded by spectrophotometer (UV-2450 PC, Shimadzu). One BAPNA unit (U ml^{-1}) trypsin was defined as the amount of enzyme required for producing an absorbance increase of 0.1 under the above conditions. Trypsin activity was calculated according to Eq. (1) * df-dilution factor

$$\text{BAPNA unit } (\text{U ml}^{-1}) = \frac{\Delta A_{410} / \min \times (\text{df})}{(0.1)} \quad (1)$$

SUPPLEMENTARY REFERENCES

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2. **Cereghino JL, Cregg JM.** 2000. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS Microbiol. Rev. **24:**45-66.