SUPPLEMENTARY INFORMATION

High-throughput enrichment of functional disulfide-rich peptides by droplet microfluidics

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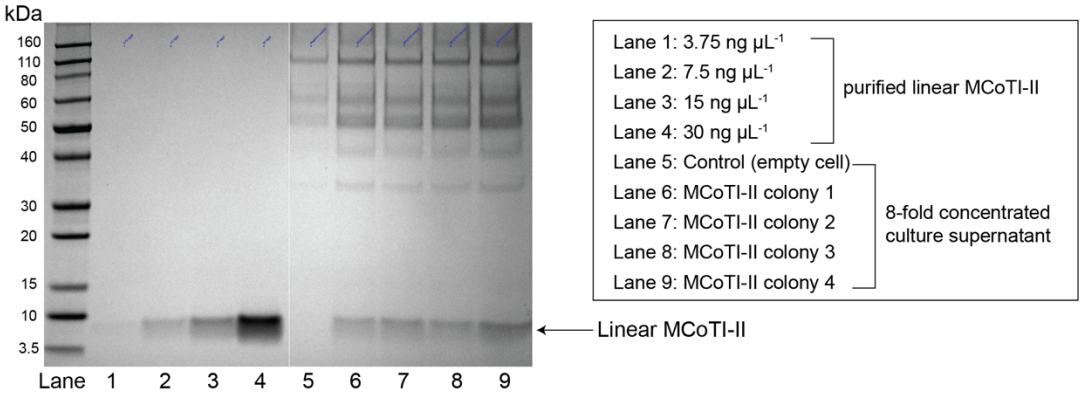
Table of Contents

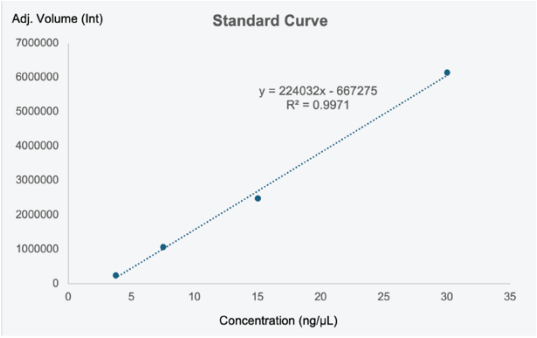
A



B

Lane	Band No.	Band Label	Mol. Wt. (KDa)	Relative Front	Adj. Volume (Int)	Volume (Int)	Abs. Quant.	Rel. Quant.	Band %	Lane %
1	1		N/A	0.924617	243746	4870212	N/A	N/A	100	5.001866
2	1		N/A	0.919717	1069144	6661820	N/A	N/A	100	14.020318
3	1		N/A	0.91716	2484861	8718574	N/A	N/A	100	32.001709
4	1		N/A	0.916963	6134952	12791529	N/A	N/A	100	61.718641
5	1		N/A	0.927467	20758	183505	N/A	N/A	100	0.656937
6	1		N/A	0.922527	973486	5631410	N/A	N/A	100	15.697303
7	1		N/A	0.928315	1031587	5930796	N/A	N/A	100	17.462416
8	1		N/A	0.934211	676882	6248693	N/A	N/A	100	14.312217
9	1		N/A	0.943645	744399	6676479	N/A	N/A	100	14.116716

C



	Adj. Volume (Int)	Conc. (ng µL-1)
MCoTI-II colony1	973486	7.32
MCoTI-II colony2	1031587	7.58
MCoTI-II colony3	676882	6.00
MCoTI-II colony4	744399	6.30

Fig. S1 Determination of the concentration of linear MCoTI-II in spent media. (A) SDS-PAGE analysis of HPLC-purified recombinant linear MCoTI-II and concentrated culture supernatant from four single colonies (~8-fold concentrated by Amicon 3K). (B) Protein gel was imaged using a Bio-Rad Gel Doc and analyzed by the Bio-Rad Image Lab software. **(**C**)** Standard curve and calculated concentration of linear MCoTI-II in concentrated supernatant.

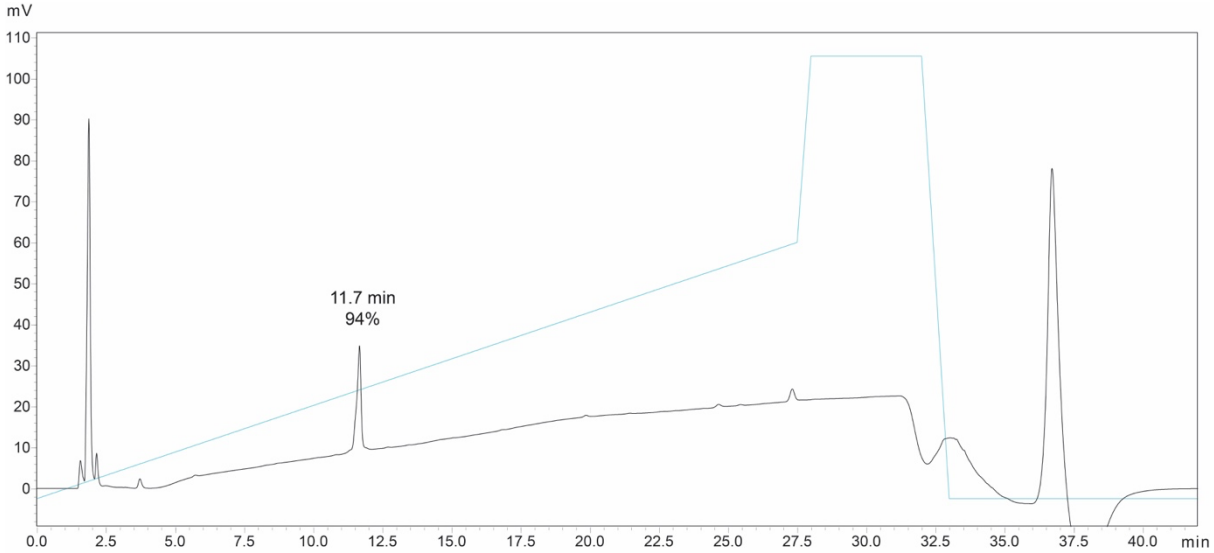


Fig. S2 RP-HPLC analysis of purified linear MCoTI-II detected via UV absorption at 214 nm. Analysis was carried out with a Phenomenex Jupiter C18 column (150 × 2 mm, 5 µm, 300 Å) at a flow rate of 0.3 mL min^{-1} and using a linear gradient of 5–60% buffer B (90% acetonitrile, 0.05% trifluoroacetic acid in water) in buffer A (0.05% trifluoroacetic acid in water) over 42 min.

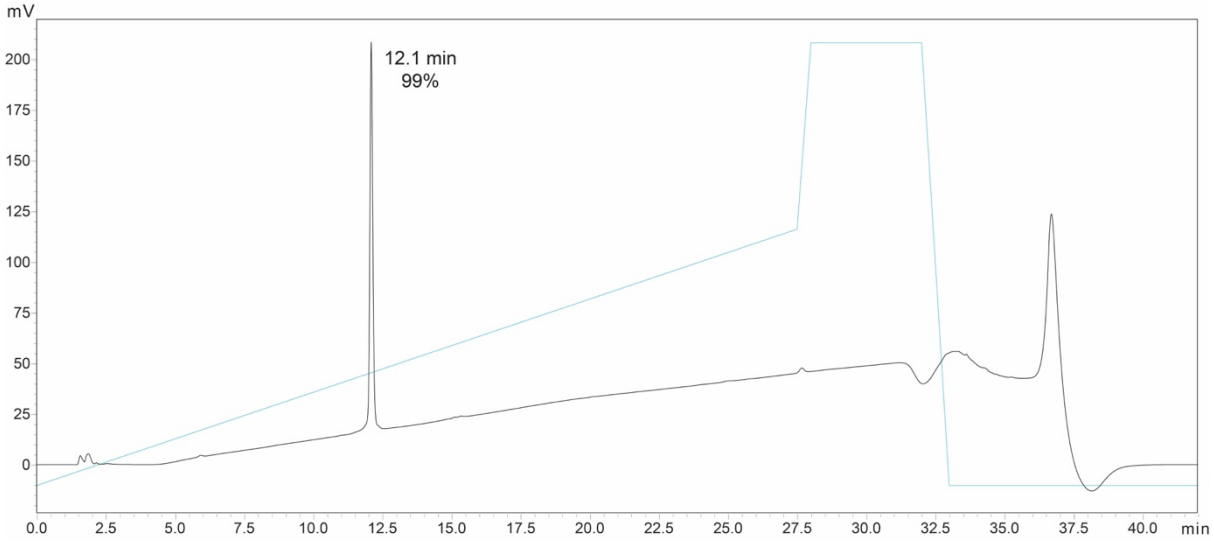


Fig. S3 RP-HPLC analysis of purified cyclic MCoTI-II via UV absorption at 214 nm. Analysis was carried out with a Phenomenex Jupiter C18 column (150 × 2 mm, 5 µm, 300 Å) at a flow rate of 0.3 mL min^{-1} and using a linear gradient of 5–60% buffer B (90% acetonitrile, 0.05% trifluoroacetic acid in water) in buffer A (0.05% trifluoroacetic acid in water) over 42 min.

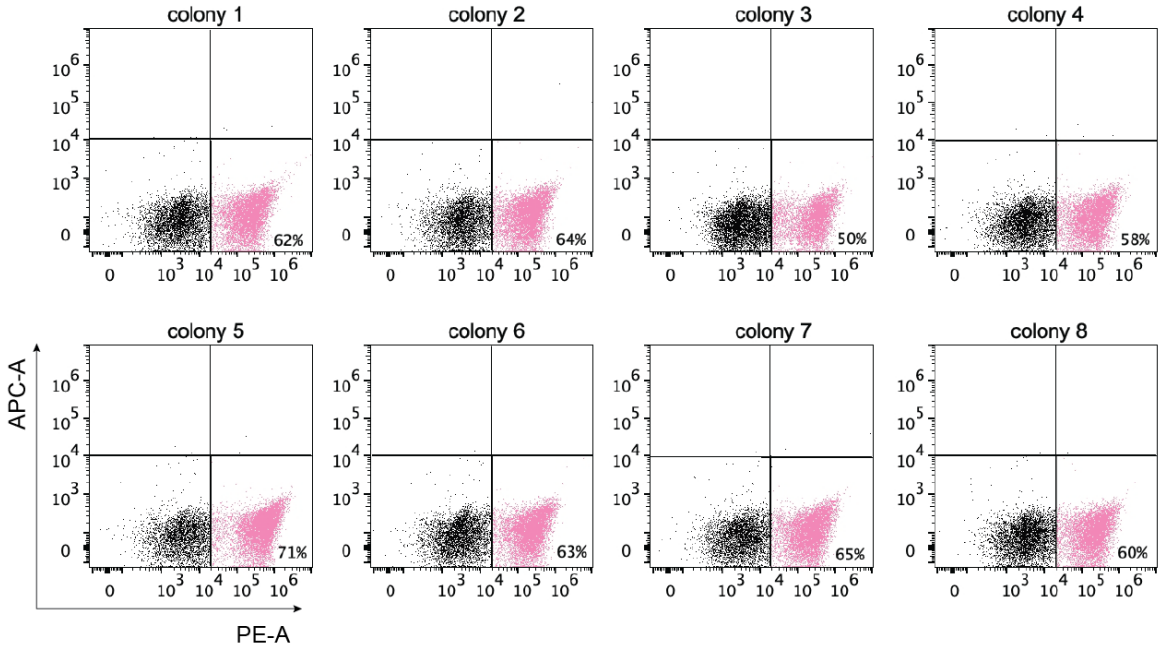


Fig. S4 Flow cytometry analysis of eight clones expressing reporter tags for protease cleavage. Eight transformants were picked from the selection plate, cultured in the liquid medium, and stained with anti-HA-PE before CytoFLEX analysis. Percentages of PE-active cells are shown in each dot plot.

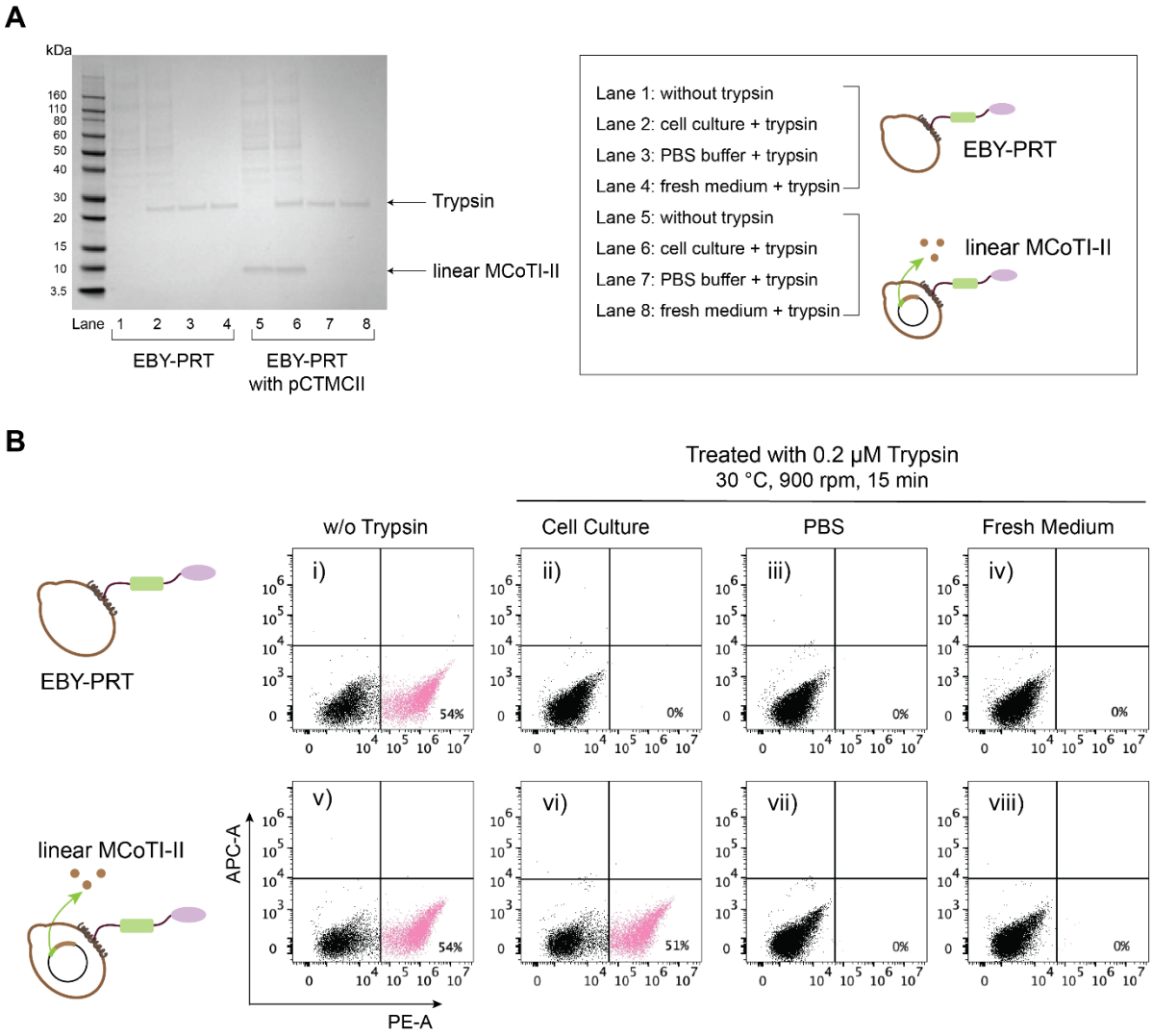


Fig. S5 Trypsin proteolysis reactions in tubes. (A) SDS-PAGE analysis of trypsin proteolysis reactions. Yeast cells (empty EBY-PRT and transformed with pCTMCII) in different conditions (original cell culture/resuspended in PBS buffer/in fresh medium) were treated without or with 0.2 μM trypsin and incubated at 30 °C for 15 min with shaking at 900 rpm. Twenty microliters of reaction supernatant were used for analysis. (B) Flow cytometry dot plots of yeast cells after staining with anti-HA-PE. First line: EBY-PRT under different conditions: i) cell culture without trypsin treatment; ii) cell culture treated with 0.2 μM trypsin; iii) cells collected with culture supernatant removed and then resuspended in PBS buffer or iv) fresh culture medium and then treated with 0.2 μM trypsin. Second line: EBY-PRT transformed with pCTMCII under different conditions v) vi) vii) viii) corresponding to i) ii) iii) iv), respectively.

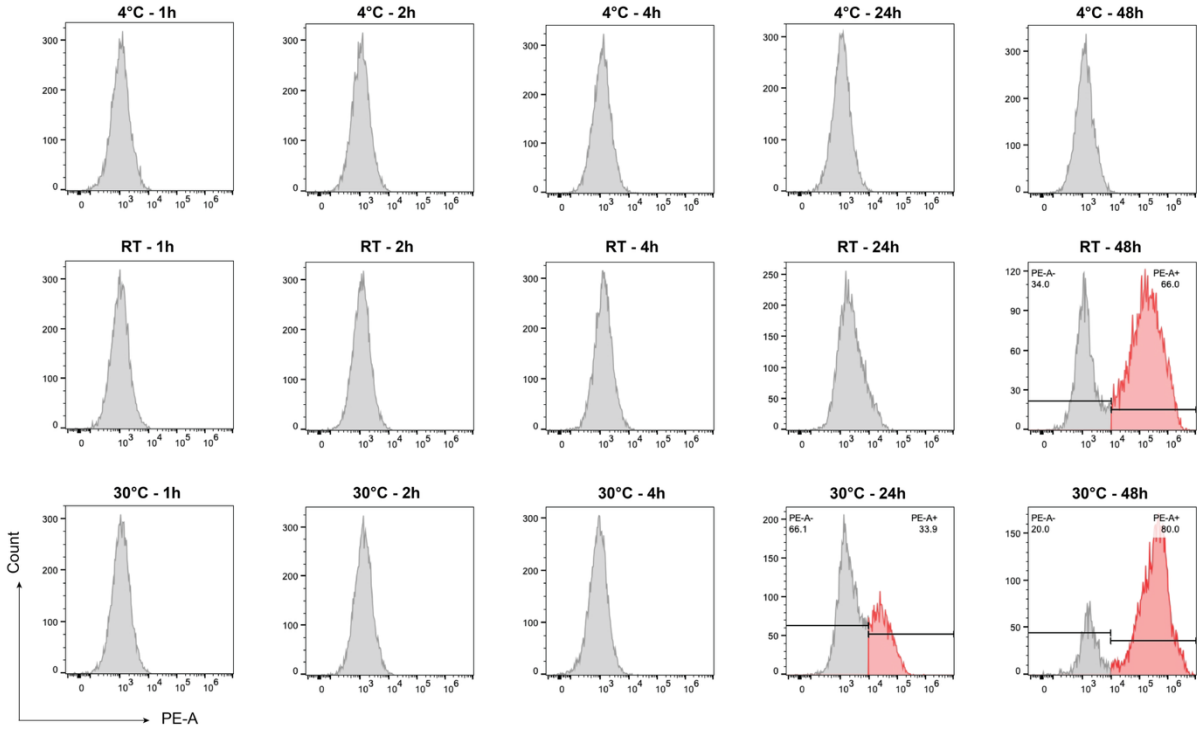


Fig. S6 Flow cytometry analysis of yeast cells (EBY-PRT transformed with pCTMCII) incubated at different temperatures (4 °C, room temperature (RT), and 30 °C). Yeast cells were induced in SDGAA medium (pH 6.85) supplemented with 0.1 μM trypsin, and collected at different time points post-induction (1 h, 2 h, 4 h, 24 h, and 48 h). Yeast cells were labeled with anti-HA-PE and analyzed by CytoFLEX.

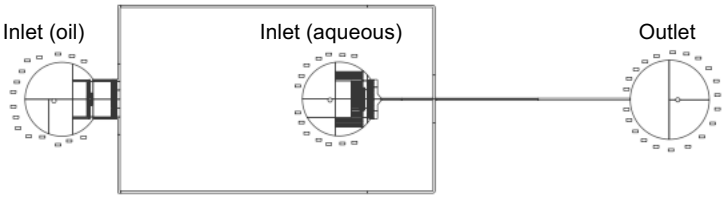


Fig. S7 Layout of the microfluidic device. The device consists of two inlets: oil (PicoSurf) and aqueous phase (cells in media). Width and height of the channel = 50 µm. Water-in-oil droplets are formed at the junction, flow out of the outlet, and can be collected in an Eppendorf or Falcon tube.

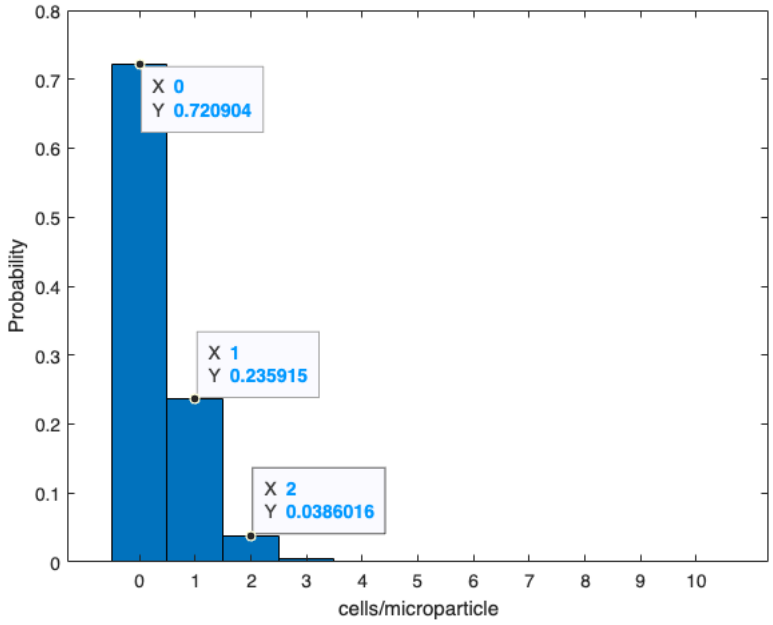


Fig. S8 The predicted probability of droplets containing different numbers of yeast cells.

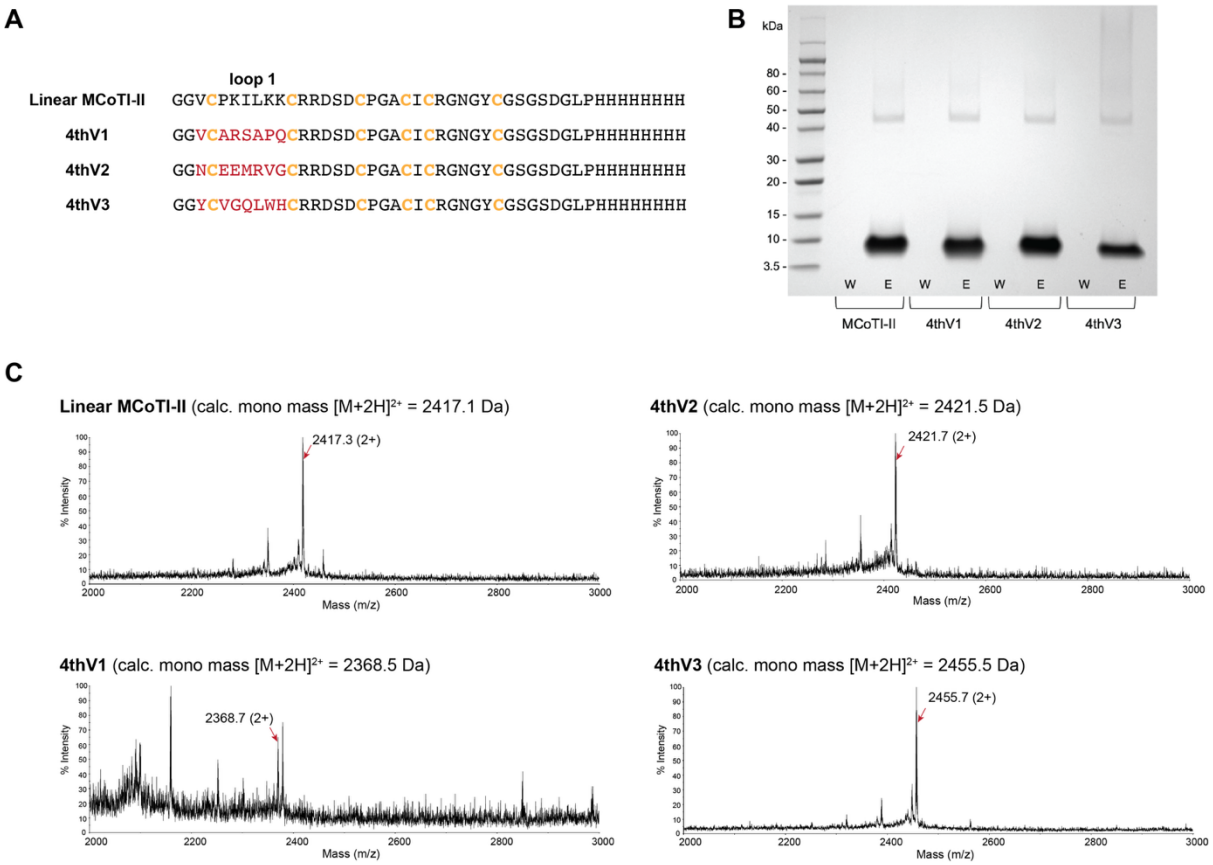


Fig. S9 Yeast secretory expression of four of the most enriched peptides from the fourth round of screening, including native MCoTI-II, 4thV1, 4thV2, and 4thV3. (A) Amino acid sequences of these four peptides. (B) SDS-PAGE analysis of IMAC fractions (W: washed fraction; E: eluted fraction) purified from 50 mL of culture supernatant. (C) MALDI-TOF-MS analysis of the eluted fractions. Observed peptide mass peaks are indicated by red arrows. All reported masses are in Dalton.

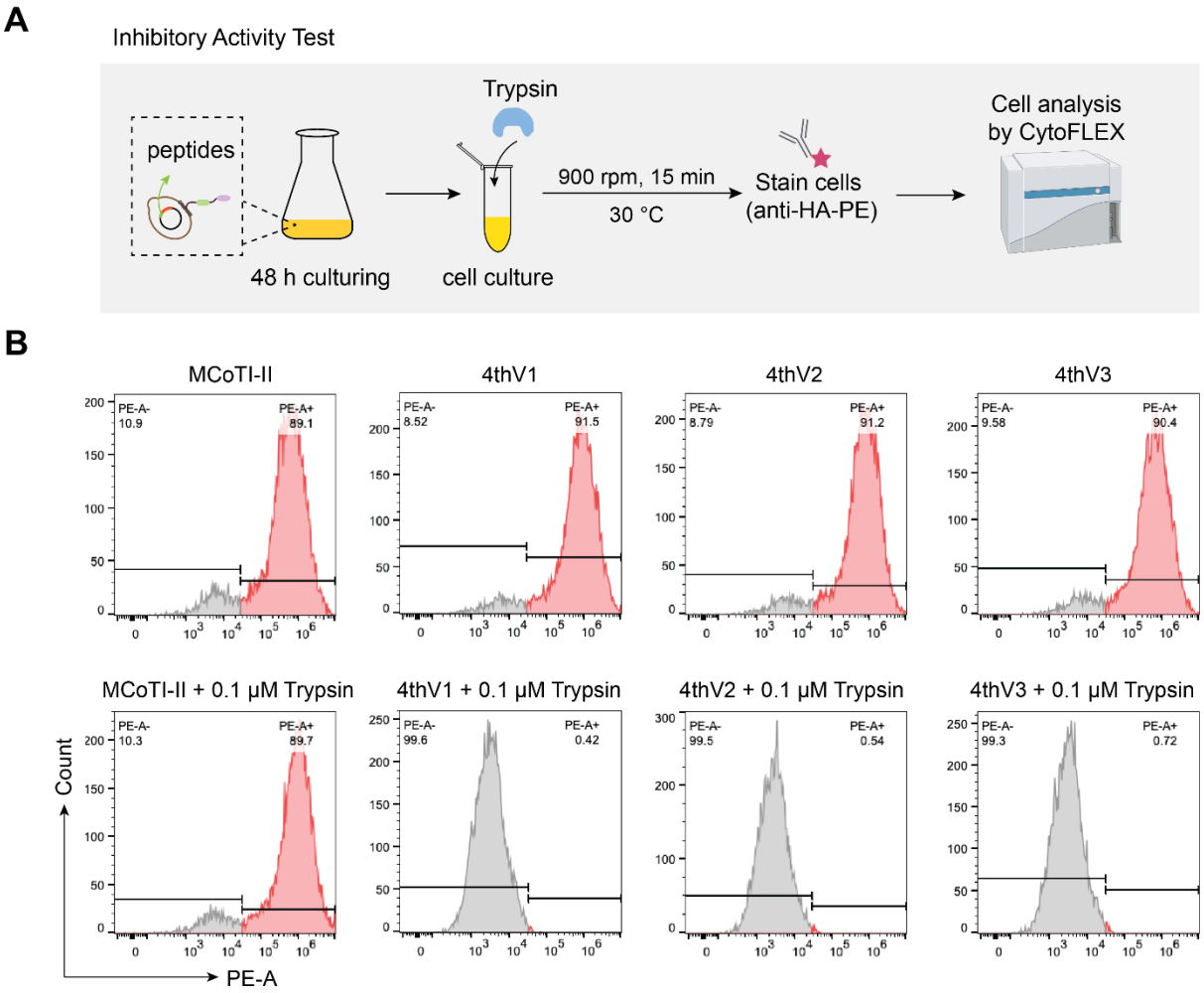


Fig. S10 Inhibitory activity test of the four enriched peptides from the fourth round of selection. (A) Illustration of the in-tube test. Yeast cells were induced and cultured for 48 h, then split in EP tubes and treated with trypsin (final concentration 0.1 μM) for 15 min before being stained with anti-HA-PE and analyzed using a CytoFLEX cytometer. (B) Histogram of flow cytometry analysis of yeast cells secreting different peptides (MCoTI-II, 4thV1, 4thV2, and 4thV3) under two conditions: without trypsin treatment and with trypsin treatment.

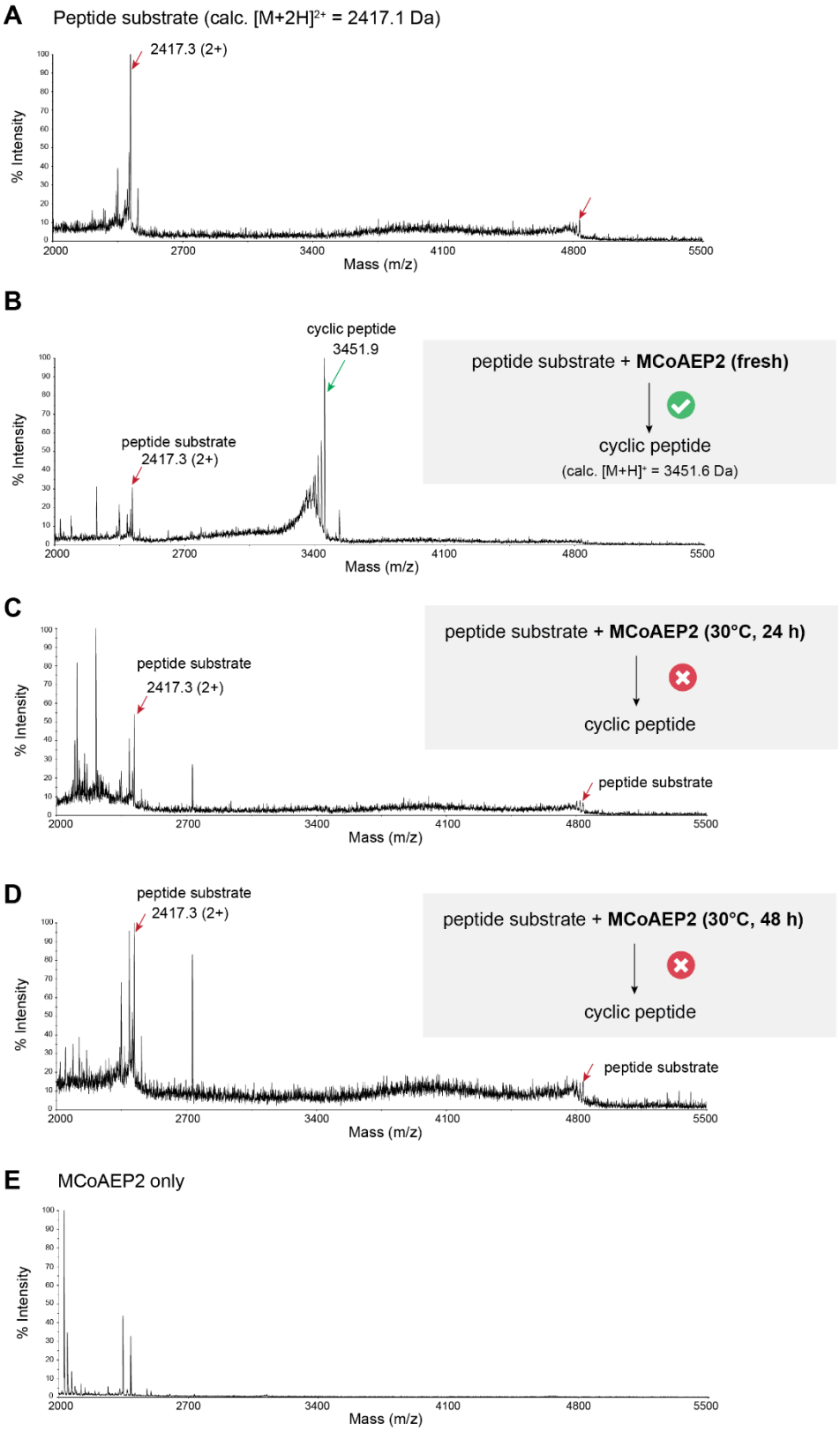


Fig. S11 MALDI analysis of MCoAEP2 efficiency on the cyclization of peptide substrate. Peptide substrate was incubated with MCoAEP2 in pH 6 buffer for 4 h at 30 °C and then quenched with 10%(v/v) formic acid before MALDI-TOF-MS analysis.

Table S1. Medium compositions

SDCAA	For one liter of medium: - 20 g D-glucose (dextrose) - 6.7 g yeast nitrogen base - 1.92 g yeast synthetic drop-out medium supplements, without tryptophan (Merk, Y1876)
SDGAA (pH 6.85)	For one liter of medium: - 20 g D-(+)-galactose - 6.7 g yeast nitrogen base - 1.92 g yeast synthetic drop-out medium supplements, without tryptophan (Merk, Y1876) pH is adjusted using 1M Na_2HPO_4 and 1M citric acid following the recipes detailed in the referenced study.[1]

Table S2. Primers for MCoTI-II library generation (5' to 3')

Linear-library-F	ttgctgctaaagaagaaggggtatctctcgagaagagaggtggtNNKtgcNNKNNKN NKNNKNNKNNKtgtaggagggattctgattg
Linear-library-R	CGTCTGAACCTGATCCACAGTAGCCATTTCCCCTGCAAATG CAGGCGCCAGGGCAATCAGAATCCCTCCTACATTTCTT
2nd Amplification-F	gggatttcgatgttgctgttttgccattttccaacagcacaaataacgggttattgtttataaatactact attgccagcattgctgctaaagaagaagg
2nd Amplification-R	tgcagagcgtagtctggaacgtcgtatgggtatctaccttcaatcgttcagtgatggtgatgatggt ggtgatgtggaagtccgtctgaacctgatccac

Two runs of PCR generated the inset DNA encoding MCoTI-II library. The first run used the primer pair of 'Linear-library-F' and 'Linear-library-R', while the second run used the primer pair of '2nd Amplification-F' and '2nd Amplification-R'.

Table S3. Primers for NGS library preparation

Nextera Overhang -F	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTATCTC TCGAGAAGAGAGGTG
Nextera Overhang -R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTgtcgtatgg gtatctaccttcaatcg
IndexAddition_F	AATGATACGGCGACCACCGAGATCTACAC(index5)ACACT CTTTCCCTACACGAC
IndexAddition_R	CAAGCAGAAGACGGCATACGAGAT(index7)GTGACTGGA GTTCAGACGTGT

Supplementary Note. Amino acid and DNA sequences used in this work.

1. pCTMCII plasmid (pCTCON2 backbone)

- α-mating factor secretion signal peptide in green
- MCoTI-II in orange
- Histag in blue

>CDS (amino acid sequence)

MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTNNG
LLFINTTIASIAAKEEGVSLEKRGGVCPKILKKCRRDSDCPGACICRGNGYCGSGSDGLPHH
HHHHHH*

>5' to 3' DNA sequence

atgagatttccttcaatttttactgctgttttattcgcagcatcctccgcattagctgctcc
agtcaacactacaacagaagatgaaacggcacaaattccggctgaagctgtcatcggttact
cagatttagaaggggatttcgatgttgctgttttgccattttccaacagcacaaataacggg
ttattgtttataaatactactattgccagcattgctgctaaagaagaaggggtatctctcga
gaagagaggtggtgtgtgccccaaaatattgaagaaatgtaggagggattctgattgccctg
gcgcctgcatttgcaggggaaatggctactgtggatcaggttcagacggacttccacatcac
caccatcatcaccatcactga

2. pCT4thV1 plasmid (pCTCON2 backbone)

- α-mating factor secretion signal peptide in green
- 4thV1 in orange
- Histag in blue

>CDS (amino acid sequence)

MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTNNG
LLFINTTIASIAAKEEGVSLEKRGGVCARSAPQCRRDSDCPGACICRGNGYCGSGSDGLPHH
HHHHHH*

>5' to 3' DNA sequence

atgagatttccttcaattttactgctgttttattcgcagcatcctccgcattagctgctcc
agtcaacactacaacagaagatgaaacggcacaaattccggctgaagctgtcatcggttact
cagatttagaaggggatttcgatgttgctgttttgccattttccaacagcacaaataacggg
ttattgtttataaatactactattgccagcattgctgctaaagaagaaggggtatctctcga
gaagagaggtggtGTTTGCGCGCGGTCGGCGCCTCAGtgtaggagggattctgattgccctg
gcgcctgcatttgcaggggaaatggctactgtggatcaggttcagacggacttccacatcac
caccatcatcaccatcactga

3. pCT4thV2 plasmid (pCTCON2 backbone)

- α-mating factor secretion signal peptide in green
- 4thV2 in orange
- Histag in blue

>CDS (amino acid sequence)

MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTNNG
LLFINTTIASIAAKEEGVSLEKRGGNCEEMRVGCRRDSDCPGACICRGNGYCGSGSDGLPHH
HHHHHH*

>5' to 3' DNA sequence

atgagatttccttcaattttactgctgttttattcgcagcatcctccgcattagctgctcc
agtcaacactacaacagaagatgaaacggcacaaattccggctgaagctgtcatcggttact
cagatttagaaggggatttcgatgttgctgttttgccattttccaacagcacaaataacggg
ttattgtttataaatactactattgccagcattgctgctaaagaagaaggggtatctctcga
gaagagaggtggtAATTGCGAGGAGATGCGGGTTGGGtgtaggagggattctgattgccctg
gcgcctgcatttgcaggggaaatggctactgtggatcaggttcagacggacttccacatcac
caccatcatcaccatcactga

4. pCT4thV3 plasmid (pCTCON2 backbone)

- α-mating factor secretion signal peptide in green
- 4thV3 in orange
- Histag in blue

>CDS (amino acid sequence)

MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTNNG
LLFINTTIASIAAKEEGVSLEKRGGYCVGQLWHCRRDSDCPGACICRGNGYCGSGSDGLPHH
HHHHHH*

>5' to 3' DNA sequence

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agtcaacactacaacagaagatgaaacggcacaaattccggctgaagctgtcatcggttact
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gcgcctgcatttgcaggggaaatggctactgtggatcaggttcagacggacttccacatcac
caccatcatcaccatcactga

5. pCfB2337-Aga2-protease cleavage site (pCfB2337 backbone)

- Aga2 in blue
- GS linker in yellow
- Protease cleavage site in green
- HA tag in violet

>CDS (amino acid sequence)

MQLLRCFSIFSVIASVLAQELTTICEQIPSPTLESTPYSLSTTTILANGKAMQGVFEYYKSV
TFVSNCGSHPSTTSKGSPINTQYVFGSGGSSGGSGSGGGSGSGGGSSGSGGGGLYAARLYAV
GGSGGSYPYDVPDYASL*

>5' to 3' DNA sequence

(TEF1 promoter Aga2 GS linker Protease cleavage site GS linker HA Tag)

ccacacaccatagcttcaaaatgtttctactccttttttactcttccagattttctcggact
ccgcgcatcgccgtaccacttcaaaacacccaagcacagcatactaaatttcccctctttct
tcctctagggtgtcgttaattacccgtactaaaggtttggaaaagaaaaaagagaccgcctc
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GCCCGACACTTGAGTCTACCCCGTATTCCTTGAGCACAACGACTATTCTGGCTAACGGGAAA
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CATCTGGTGGCTCTGGTTCAGGCGGAGGTAGTGGAAGTGGCGGCGGCTCATCAGGTAGCGGC
GGAGGCGGTTTATATGCCGCAAGGCTATATGCAGTTGGCGGTAGCGGCGGCTCCTATCCTTA
CGATGTTCCAGATTATGCGAGCCTGTAA

Supplementary Reference

1 R. C. Prins and S. Billerbeck, *BMC Microbiol.*, 2021, **21**, 1-9.