Supplementary Material

Characterization and Application of Natural and Recombinant Butelase-1 to Improve Industrial Enzymes by End-to-end Circularization

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The Supplementary information contains:

- Materials and Methods
- One table and 11 Figures supporting the results described in the main text.

Materials and Methods

Materials

All chemical compounds were purchased from Sigma-Aldrich. Trypsin was purchased from Pierce (MS grade, Thermo Scientific). Peptide substrates: kB1-NHV (GLPVCGETCVGGTCNT PGCTCSWPVCTRN-HV), GN14-GI (GISTKSIPPISYYN-GI), GN14-HV (GISTKSIPPISYYN-HV), were synthesized by GL Biochem (Shanghai, China). All plant samples of *C. ternatea* were collected in Nanyang Technological University herbal garden.

Tissue-specific screening of butelase-1 activity

Each part of C. ternatea was ground in liquid nitrogen into fine power and extracted with 1:10 (w/v) of extraction buffer (50 mM sodium phosphate buffer, 1 mM ethylenediamine-tetraacetic acid (EDTA), 1 mM β -mercaptoethanol (β -ME), 1 mM *phenylmethylsulfonyl fluoride* (PMSF), pH 6.0). The tissue extracts were mixed at 1:1 (v/v) ratio with 200 μ M solution of kB1-NHV and incubated at 37°C for 10 min. The reactions were quenched with HCl to pH 2.0 and analyzed by MALDI-TOF MS (Applied Biosystems, Sciex TOF/TOFTM 5800).

Extraction and purification of butelase-1 from C. ternatea

100 g of young shoot of C. ternatea was frozen in liquid nitrogen, blended in 1/10 (w/v) extraction buffer with 2% w/w polyvinylpolypyrrolidone. Plant extract was clarified by passing through a piece of cheesecloth followed by centrifugation at 15,000 g, 4 °C for 20 min. The clarified supernatant was collected and applied onto an anion-exchange flash chromatographic column packed with 100 mL of Q Sepharose Fast Flow resin (GE Healthcare). The column was washed with elution buffer EB1 (50 mM sodium phosphate buffer, 1 mM EDTA and 5 mM β -ME, pH 6.0) and eluted using 1000 mL of elution buffer EB2 (EB1 with 0.1 M NaCl). The eluent was dialyzed against 4 L of EB2 for 4 h, and 12 L of EB2 overnight at 4°C. The dialyzed eluent was fractioned by three 5 mL HiTrap Q HP columns (GE Healthcare) connected in serial. Fractions were screened for cyclase activity and active fractions were pooled. The sample was concentrated by 10-kDa filter concentrator (Vivaspin Turbo 15, Sartorius) and further purified by a HiLoad Superdex 75 g 16/60 prep grade column (GE Healthcare). Active fractions were subjected to analytical anion-exchange HPLC purification (200 × 4.6 mm, PolyWax LP, PolyLC) to obtain pure butelase-1. The purity of isolated butelase-1 was confirmed by SDS-PAGE. Protein concentration was determined by Bradford assay and A280 absorption using Nanodrop.

Bacterial expression and purification of butelase-1, phytase and lipase

Butelase-1 cDNA sequences Opt-Bu1(Δ SP-Ubi) (optimized codons), Opt-Bu1(Δ 1-25) (optimized codons), Bu1(Δ SP) (native codons), and Bu1(Δ 1-25) (native codons) were cloned into pET28a(+) with NdeI/XhoI restriction enzymes in-frame with the N-terminal His6 tag. Plasmids were transformed into SHuffle T7 E. coli competent cells (NEB, C3029J). Overnight-grown culture was amplified in 1 L LB broth with kanamycin until OD₆₀₀ reached 0.4. Cells were then induced with 0.1 mM IPTG at 16 °C for 24–48 h. cDNA of phytase-NHV was synthesized by GenScript with N-terminal His6-TEV tag (Fig. S10) and subcloned into pET28a and expressed by E. coli (DE3) Rosetta with 0.5 mM IPTG induction at 16 °C for 24 h. cDNA of lipase T1.2-NHV was synthesized by GenScript with N-terminal His6-TEV tag (Fig. S11) and subcloned into pET30a for recombinant expression by E. coli (DE3) Rosetta with 0.1 mM IPTG induction at 16 °C overnight.

Bacterial cells were harvested by centrifugation at 6000 g for 15 min at 4 °C. Cell pellets were resuspended with cold lysis buffer (50 mM Na HEPES, 0.1 M NaCl, 1 mM EDTA, 5 mM β -mercaptoethanol, 0.1% TritonX-100 at pH 7.5) in the ratio of 10 mL per 1 g. Cells were lysed by sonication on ice. Clarified cell lysate was obtained by centrifugation at 12,000 g for 15 min. cOmpleteTM His-Tag Purification Resin (Roche) were equilibrated with cold binding buffer

(HEPES, 0.1 M NaCl, 1 mM EDTA, 5 mM β-ME, pH 7.5). For the clarified lysate from 1 L culture, 0.1 mL equilibrated beads were added and the container was rolled gently for 1 h at 4 °C for affinity binding. Beads were loaded to centrifuge columns equipped with polyethylene filters and washed with 2x bead-volume (BV) washing buffer (50 mM HEPES, 50 mM imidazole, 0.1 M NaCl, 1 mM EDTA, 5 mM β -ME, pH 7.5) for four times. Beads were then soaking with 2x BV elution buffer (50 mM HEPES, 500 mM imidazole, 0.1 M NaCl, 1 mM EDTA, 5 mM β-mercaptoethanol, pH 7.5) on ice for 5 min and span for 0.5 min at 1,000 g at 4 °C. Elution was repeated for four times. For butelase-1 proenzymes, the eluted proteins were concentrated in a centrifugal filter (Vivaspin 500, 10 kDa MWCO, Sartorius) and buffer-exchanged with enzyme storage buffer (20 mM sodium phosphate, 0.1 M NaCl, 1 mM EDTA, 5 mM β-ME, 20% sucrose, pH 6.0). For recombinant PhyC, the eluted proteins were concentrated and buffered exchanged with 50 mM Tris-Cl buffer (pH 7.5) containing 2 mM CaCl₂. For recombinant T1.2, the eluted proteins were buffer exchanged with TEV buffer (0.1M NaCl, 20mM Tris, 10% Glycerol (v/v), pH7.5) and then subjected to TEV protease (in-house expressed, carry N-terminal His6-tag) cleavage at 30 °C for 2 h. Digestion mixture was passed through IMAC column again to obtain purified GV-T1.2-NHV in the flow through. Ion-exchange and size-exclusion FPLC were performed when the protein purity was not satisfactory after IMAC. Protein concentration was determined by Bradford assay and A280 absorption using Nanodrop.

Acid-induced auto-activation of recombinant butelase-1 proenzymes

The recombinant butelase-1 proenzyme solution (10-20 μ M) in enzyme storage buffer was acidified to pH 4.0-4.5 with 0.2 M citric acid and incubated at 4, 25, or 37 °C for 30 min to 16 h. The progress of activation at different time points was analyzed by SDS-PAGE (Fig. S4). The auto-activation procedure was quenched by bringing pH back to 6.0 with 0.2 M Na2HPO4 buffer on ice.

In-gel trypsin digestion and LC-MS/MS sequencing of butelase-1

The purified natural and recombinant butelase-1 were subjected to SDS-PAGE and the targeted bands were cut into thin slice for in-gel digestion. The reduction and alkylation of disulfide bonds was performed in one-pot by addition of 5 mM DTT and 10 mM 2-iodoacetamide in a 1 M Tris-HCl, pH 8.6, at 55 °C for 30 min. Tryptic digestion was performed with 10 µg/mL trypsin (Pierce, MS grade, Thermo Scientific) at pH 7.8 and 37 °C overnight. Digested peptides were extracted from gel pieces with 50% acetonitrile/0.1% formic acid and dried by SpeedVac concentrator (Thermofisher) at room temperature. Dried peptide fragments were re-dissolved in 1% formic acid and subjected to LC-MS/MS sequencing on a Dionex UltiMate 3000 UHPLC/Orbitrap Elite LC-MS system (Thermo Scientific Inc., Bremen, Germany). The resulting data were analyzed using PEAKS Studio (software version 7.5, Bioinformatics Solutions, Waterloo, Canada) with 10 ppm MS and 0.05 Da MS/MS tolerances.

Activity comparison of natural and recombinant butelase-1

The activity comparison of nBu1 and rBu1 was performed with 20 nM enzyme and 20 μ M synthetic peptide substrate GN14-HV in the reaction buffers (20 mM sodium citrate or sodium phosphate buffers containing 1 mM EDTA, 1 mM DTT, with pH ranging from pH 4.5 to 8) at 25 °C, 37 °C or 42 °C for 5 min. Reaction was quenched with equal volume of 2-propanol and subjected to mass spectrometry analysis using MALDI-TOF MS. NaCl concentrations ranging from 0 to 1.5 M were used to determine the effect on cyclization activity of butelase-1 at different ionic strength in the same pH 6.0 reaction buffer as above. The product yields were calculated by the peak areas determined using Data Explorer software by the equation [% cyclization] = Area (cGN14)/Area (cGN14 + GN14-HV). The areas of peaks carrying sodium or potassium ion adducts were also counted.

Thermal shift assay

Thermal stability of natural and recombinant butelase-1, linear and circular phytase and lipases were measured by a ThermoFluor assay conducted in a Real-Time PCR Detection System (Bio-Rad) with a linear temperature gradient from 25 to 85 °C in 60 min.³⁷ For each well, 1 µg enzyme and 8× SYPRO orange fluorescent dye (Thermo Fisher Scientific) were added. Butelase-1 buffers (20 mM sodium phosphate, 5 mM β -ME) with pH ranging from 5 to 8, phytase buffer (50 mM Tris-Cl, 2 mM CaCl2, 0.15 M NaCl, pH 7.5), and lipase buffer (50 mM Tris-Cl, pH 8.0) were used to top-up the solution to 25 µL in each well. The melting temperature (Tm) was calculated by plotting the change of RFU per degree (-d(RFU)/dT) against temperature.

Evaluation of butelase-1 storage conditions

Butelase-1 solution (20 μ l of 10 μ M in enzyme storage buffer (containing 20% sucrose, pH 6.0) was froze using liquid nitrogen and kept at -80°C freezer or directly froze in -20 °C freezer. Frozen enzyme stocks were thawed on ice (0 °C). Samples without 20% sucrose were rest at 4 °C overnight after thawed before the activity tests. The freeze-thaw cycle was repeated for 5 times. Activity was measured after each cycle by comparing the cyclization efficiency using GN14-HV as the model substrate.

Butelase-1 lyophilization solutions were prepared with formulas listed in Table 1 in 20 mM sodium phosphate buffer (with 5 mM β -ME, 0.1 M NaCl, pH 6.0). The freeze-dried powders were stored at room temperature for 5 days before reconstituting in 5 mM fresh β -mercaptoethanol (β -ME) solution. Activity of lyophilized butelase-1 were examined by the cyclization reactions as stated above.

Cyclization of phytase and lipase using soluble and immobilized butelase-1

Linear precursor ML-phytase-NHV was cyclized by soluble butelase-1 in a pH 6.0 sodium phosphate buffer with an enzyme-to-substrate ratio of 1:100 at 37 °C for 30-60 min to reach >90% completion. The cyclized phytase was purified by ion-exchange FPLC using a HiTrap Q HP column (1 mL, Merck).

Linear precursor GV-T1.2-NHV was cyclized using immobilized conA-nBu1 beads prepared as previously reported.³⁷ The effective concentration of butelase-1 on ConA-nBu1 beads in this work was 4 μ M. GV-T1.2-NHV was diluted to 40 μ M by reaction buffer (20 mM sodium phosphate, 5 mM β -ME, pH 7.0) and mixed with ConA-Bu1 beads in a ratio of 50:1 v/v to give a 1:500 enzyme-to-substrate molar ratio. Reaction was performed at 25 °C with gentle rocking of the reaction tube for 30 min and solution containing circular lipase T1.2 was separated from beads and further purified by ion-exchange FPLC.

Activity tests for lipases

Linear and circular lipases T1.2 were examined for their esterase activity using *p*-nitrophenyl phosphate (pNPP) as described by Guo et al. ³⁹ A mixture of 1 µmol pNPP and 1 nmol lipases in 50 mM Tris-Cl buffer (pH 9.0) was reacted at room temperature and quenched using 2% SDS after 3 min to obtain the initial catalytic rate. Reaction was then diluted 10x with Tris-Cl buffer and the amount of p-nitrophenol was determined by its maximum absorbance at 405nm (Implen, USA) immediately. Samples contained only pNPP and reaction buffer were used as negative control.

Activity tests for phytases

Linear and circular phytases were subjected to phytate digestion assay with 0.2% w/v sodium phytate (Sigma) solution in 10 mM Tris-Cl buffer (2 mM CaCl₂, pH 7.5). Reactions were performed at 50-95 °C, at pH 7.5 for 30 min and quenched with equal volume of trichlroacetic acid (5% w/v). The amount of released inorganic phosphates were measured by adding 4x volume of the freshly prepared coloring reagent (1.5% w/v ammonium molybdate, 5.5% w/v sulfuric acid, and 2.7% w/v ferrous sulfate)³⁸ into the quenched reaction mixture and the production of phosphomolybdate was measured at 700 nm. For heat tolerance assays, phytases were heated at 50-95 °C for 5 min and

then incubate at room temperature for 1 h to renature. Phytase activity tests were performed with the treated enzymes using the same protocol as above.



Fig. S1 Butelase-1 activity screening of tissue extracts. 10 μ L of tissue extracts were mixed with 10 μ L substrate solution (0.2 mM oxidized kB1-HV in pH 6 reaction buffer) and incubated at 37 °C for 10 min. The reaction products were analyzed by MALDI-TOF mass spectrometer in reflectron-mode.



Fig. S2 Purification of natural butelase-1. (A) Anion-exchange chromatography profile (B) Size-exclusion chromatography profile. Fractions containing butelase-1 were determined by conducting the cyclization reaction of GN14-HV.

cBul nBul	CTGCGTGGTGGCATTC GGTATCC ** ** *	CGTGACGATTTTCTGCGTCTG CGTGACGATTTTCTCCGATTG **************	CCGAGCCAAGCGAGCAAGT CCCTCCCAAGCTTCCAAGT ** ***** *****	TCTTTCAGGCGGACGAT. TCTTCCAAGCAGACGAC	AACGTTGAAGGTACCCGTTGGGCGGTGC AACGTTGAGGGGACCAGGTGGGCTGTCT	IGGTTGCGGGTAGCAAAGGT 120 TAGTCGCCGGTTCCAAGGGC 111 * ** ** *** *** ***
	L R G G I	RDDFLRL	PSQASK	FFQADD	NVEGTRWAV	L V A G S K G
cBul nBul	TACGTGAACTATCGTC TACGTCAATTACAGGC ***** ** ** ** *	CACCAAGCGGACGTTTGCCAC CATCAGGCTGATGTTTGTCAT ** ** ** ** ** *****	GCGTATCAGATCCTGAAGA GCATATCAAATTCTGAAGA ** ***** ** *******	AAAGGTGGCCTGAAGGAT AAAGGTGGCTTGAAAGAT	GAGAACATCATTGTGTTTATGTACGACG. GAAAACATTATTGTATTTATGTATGATG. ** ***** ***** *****************	ATATTGCGTATAACGAAAGC 240 ATATTGCCTACAATGAATCG 231 ******* ** ** ***
	Y V N Y R	H Q A D V C H	A Y Q I L K	K G G L K D	E N I I V F M Y D	DIAYNES
cBul nBul	AACCCGCACCCGGGCG AACCCGCATCCAGGAG ******** ** ** **	GTTATCATTAACCACCCGTAC GTAATTATTAACCACCCATAT ** ** *************	GGCAGCGACGTGTATAAAG GGAAGTGATGTTTACAAAG ** ** ** ** ** ** *****	GGTGTTCCGAAGGACTAC GGAGTCCCTAAGGATTAC	GTGGGTGAAGATATCAACCCGCCGAACT GTCGGTGAAGACATTAATCCTCCCAACT ** ******** ** ** ** ** ** **	TCTATGCGGTTCTGCTGCGG 360 TTTATGCTGTTCTACTTGCA 351 * ***** *****
	N P H P G	V I I N H P Y	G S D V Y K	G V P K D Y	V G E D I N P P N	FYAVLLA
cBul nBul	AACAAAAGCGCGCTGA AATAAGTCTGCTCTTA ** ** ** ** **	ACCGGTACCGGTAGCGGCAAG ACTGGTACTGGTAGCGGCAAG ** ***** ************	GTGCTGGACAGCGGTCCGA GTTTTGGACAGTGGTCCCA ** ****** *****	ACGATCACGTTTTTATC ATGATCATGTATTTATA ** ***** ** *****	FACTATACCGATCACGGTGGCGCGGGGCG FACTACACTGACCATGGAGGTGCAGGAG ***** ** ** ** ** ** ** ** ** **	TGCTGGGTATGCCGAGCAAG 480 TACTTGGGATGCCTTCTAAG 471
	N K S A L	T G T G S G K	V L D S G P	N D H V F I	YYTDHGGAG	V L G M P S K
cBul nBul	CCGTACATTGCGGCGA CCATACATTGCTGCGT ** ******** ***	AGCGACCTGAACGATGTTCTG. FCTGATCTGAATGATGTTTTG ** ***** ****** **	AAGAAAAAGCACGCGAGCG AAGAAGAAGCATGCTTCTG ***** ***** ** ** *	GGTACCTACAAAAGCATC GGAACATATAAAAGCATA	STGTTCTATGTTGAGAGCTGCGAAAGCG STATTTTATGTAGAGTCTTGTGAATCCG ** ** ***** *** ** ** ** **	GCAGCATGTTTGACGGTCTG 600 GGAGTATGTTTGATGGTCTT 591 * ** ******
	P Y I A A	S D L N D V L	K K K H A S	G T Y K S I	V F Y V E S C E S	GSMFDGL
cBul nBul	CTGCCGGAGGATCACA CTTCCTGAAGACCATA	AACATTTATGTGATGGGCGCG. AATATCTATGTAATGGGAGCT ** ** ***** ***** **	AGCGACACCGGTGAAAGCA TCAGATACAGGAGAAAGCA ** ** ** ********	AGCTGGGTTACCTACTGC AGTTGGGTAACATATTGT	CCGCTGCAACATCCGAGCCGCCGCCGC CCTTTACAGCATCCTAGCCTCCTCCAG. ** * ** ***** ***** ** **	AGTATGATGTGTGGCGTTGGC 720 AATATGATGTCTGCGTCGGT 711 * ******** ***** **
	LPEDH	N I Y V M G A	S D T G E S	S W V T Y C	PLQHPSPP	EYDVCVG
cBul nBul	GACCTGTTCAGCGTGG GACCTCTTCAGTGTTG ***** ***** ** *	GCGTGGCTGGAAGACTGCGAT GCTTGGTTGGAAGACTGTGAT ** *** ***********	GTTCACAACCTGCAGACCG GTACACAATTTGCAAACAG ** ***** **** ***	GAGACCTTTCAGCAACAG GAAACTTTCCAACAGCAA ** ** ** ** ** **	FACGAAGTGGTTAAAAACAAGACCATCG FATGAAGTGGTAAAAAATAAGACCATCG ** ******** ***** *****	TGGCGCTGATTGAGGACGGT 840 TGGCACTAATAGAAGATGGT 831 **** ** ** ** **
	D L F S V	A W L E D C D	V H N L Q T	E T F Q Q Q	Y E V V K N K T I	VALIEDG
cBul nBul	ACCCACGTGGTTCAAT ACCCATGTTGTGCAGT ***** ** ** ** **	FACGGCGATGTGGGTCTGAGC. FATGGTGACGTAGGACTTAGC. ** ** ** ** ** ** ** **	AAGCAGACCCTGTTCGTTT AAACAGACTCTCTTCGTAT ** ***** ** ****** *	PATATGGGCACCGACCCG PATATGGGTACAGATCCT	SCGAACGATAACAACACCTTTACCGACA. SCTAATGATAATAATACTTTTACGGACA. ** ** ***** ** ** ******	AAAACAGCCTGGGTACCCCG 960 AAAATTCATTAGGGACACCT 951 **** * ** **
	T H V V Q	Y G D V G L S	K Q T L F V	Y M G T D P	A N D N N T F T D	K N S L G T P
cBul nBul	CGTAAGGCGGTTAGCC AGAAAAGCAGTTAGTC * ** ** ***** **	CAACGTGACGCGGATCTGATC CAACGGGATGCAGATCTCATC	CACTACTGGGAGAAATATC CATTACTGGGAAAAGTATC * ******** ** *****	CGTCGTGCGCCGGAAGGT. CGTAGAGCTCCTGAGGGT	AGCAGCCGTAAGGCGGAGGCGAAAAAGC. FCTTCTAGGAAAGCTGAAGCTAAGAAAC. * ** ** ** ** ** ** ** **	AGCTGCGTGAAGTGATGGCG 1080 AACTTCGGGAAGTAATGGCT 1071 ** ** *****
	R K A V S	Q R D A D L I	H Y W E K Y	R R A P E G	S S R K A E A K K	QLREVMA
cBul nBul	CACCGTATGCACATCG CACAGAATGCATATAG *** * ***** ** *	GACAACAGCGTTAAACACATT GACAACAGTGTGAAACACATT	GGCAAGCTGCTGTTCGGCA GGAAAGCTCTTATTTGGCA ** ***** * ** ****	ATCGAAAAAGGTCACAAG. ATTGAAAAGGGTCATAAA.	ATGCTGAACAACGTGCGTCCGGCGGGTC ATGCTCAACAATGTTAGACCTGCAGGGC ***** ***** ** * * ** ** ** **	TGCCGGTGGTTGACGATTGG 1200 TACCAGTTGTAGATGACTGG 1191 * ** ** ** ** ** ***
	H R M H I	D N S V K H I	GKLLFG	I E K G H K	M L N N V R P A G	LPVVDDW
cBul nBul	GATTGCTTCAAAACCC GATTGCTTCAAAACAC	CTGATTCGTACCTTTGAGACC CTGATTAGGACTTTTGAGACA	CACTGCGGCAGCCTGAGCG CATTGTGGATCCCTGTCAG ** ** ** ** **** *	GAATACGGTATGAAGCAC. GAGTATGGTATGAAACAT.	ATGCGTAGCTTTGCGAACCTGTGCAACG ATGCGGTCGTTTGCAACTTGTGCAACG	CGGGTATCCGTAAAGAGCAA 1320 CTGGAATACGGAAAGAGCAA 1311 * ** ** ** *********
	D C F K T	L I R T F E T	H C G S L S	E Y G M K H	M R S F A N L C N	AGIRKEQ
cBul nBul	ATGGCGGAAGCGAGCG ATGGCTGAGGCCTCTG ***** ** ** **	GCGCAGGCGTGCGTTAGCATT GCACAAGCTTGCGTCAGTATT ** ** ** ****** ** ***	CCGTAA CCTGATAATCCATGGAGTT **	CTCTACACGCTGGTTTC.	AGTGTATAG	
	MAEAS	A Q A C V S I	P D N P W S	S L H A G F	SV *	

Fig. S3 Sequence alignment of codon-optimized (cBu1) and native butelase-1 cDNA (nBu1) without the signal peptide. Letters in orange, sequence from the N-terminal ubiquitin-tag.



Fig. S4 Acid-induced auto-activation of recombinant butelase-1. Activation conditions changed from mild to strong from left to right panels. Samples in the left gel started with 1 mg/mL protein concentration. Samples in the right gel started with 0.5 mg/mL protein concentration.



Fig. S5 LC-MS/MS analysis of autoactivation sites of butelase-1. (A) Cleavage sites of natural butelase-1 are N-terminus after Q37, D40, N41 and C-terminus after N322, D323, and N331. (B) Cleavage sites of recombinant butelase-1 are N-terminus after Q37, C-terminus after N331. The C-terminal processing site of the intermediate was predicted to be N403 after α 8 helix based on the band size in SDS-PAGE gel. The region including α 7 and α 8 are K/R-rich and thus the very short tryptic digested peptides may be missing after desalting. Proteomic data was processed by PEAKs Studio. Blue lines represents peptides identified by comparing with the template amino acid sequence. Color squared letters codify for identified post-translational or chemical modifications.



Fig. S6 Determination of the major glycosylation site in the natural butelase-1 by LC-MS/MS analysis. (A) Fragments identified by PEAKs in the glycosylated region. Blocks with "n" or "h" indicates side-chain modifications predicted to be glycosylation. (B) MS/MS profile of one peptide fragment with a 1216.42 Da glycan at the side chain of Asn94.

Name	Organism	PDB code	Resolution	Asp/Snn
hLEG with YVAD-CMK	Homo sapiens	4AWA	2.50 Å	Asp
hLEG with AAN-CMK	Homo sapiens	4AWB	2.70 Å	Asp
hLEG with YVAD-CMK	Homo sapiens	4AW9	2.2 Å	Asp
CgLEG	Cricetulus griseus	4D3X	1.848 Å	Snn
CgLEG (proenzyme)	Cricetulus griseus	4D3Y	2.4 Å	Snn
CgLEG (proenzyme)	Cricetulus griseus	4D3Z	2.3 Å	Snn
hLEG (proenzyme)	Homo sapiens	4FGU	3.90 Å	Asp
mLEG at pH 4.5	Mus musculus	4NOM	2.006 Å	Asp
mLEG at pH 3.5	Mus musculus	4NOJ	2.8 Å	Asp
mLEG at pH 7.5	Mus musculus	4NOK	2.5 Å	Asp
mLEG-D233A at pH 7.5	Mus musculus	4NOL	2.7 Å	Asp
hLEG with Cystatin E/M	Homo sapiens	4N6N	1.87 Å	Snn
hLEG with Cystatin E/M	Homo sapiens	4N6O	1.8 Å	Snn
OaAEP1b (proenzyme)	Oldenlandia affinis	5H0I	2.56 Å	Asp
hLEG with compound 11	Homo sapiens	5LUA	2 Å	Snn
hLEG with compound 11b	Homo sapiens	5LUB	2.1 Å	Snn
hLEG with YVAD-CMK and compound 11	Homo sapiens	5LU9	2.27 Å	Snn
hLEG with compound 11b	Homo sapiens	5LU8	1.95 Å	Snn
AtLEGy in two-chain form (proenzyme)	Arabidopsis thaliana	5NIJ	2.75 Å	Snn
AtLEGy with YVAD-CMK	Arabidopsis thaliana	5OBT	1.5 Å	Snn
VcAEP (proenzyme)	Viola canadensis	5ZBI	2.09 Å	Asp
HaAEP with AAN	Helianthus annuus	6AZT	1.8 Å	Snn
butelase-1 (proenzyme)	Clitoria ternatea	6DHI	3.1 Å	Snn
VyPAL2 (proenzyme)	Viola yedoensis	6IDV	2.4 Å	Snn
butelase-2-N373A (proenzyme)	Clitoria ternatea	6L4V	1.35 Å	Snn
butelase-2-G252V (proenzyme)	Clitoria ternatea	6L4W	1.66 Å	Snn
butelase-2-G182A/P183A/G252V (proenzyme)	Clitoria ternatea	6L4X	2.644 Å	Snn
butelase-2-G252V (C222-oxidized, proenzyme)	Clitoria ternatea	6L4Y	1.5 Å	Snn
butelase-2- P183A/G252V (proenzyme)	Clitoria ternatea	6LKO	2 Å	Snn
CeAEP (proenzyme)	Canavalia ensiformis	6XT5	2.69 Å	Snn
AtLEGβ (proenzyme)	Arabidopsis thaliana	6YSA	2.01 Å	Snn

 Table S1
 List of crystal structures of AEPs and PALs and the presence of Asp-to-Snn conversion in these structures.

A Asp-to-Snn conversion

C MS/MS fragmentation of peptides containing Asp164



Fig. S7 Confirmation of Asp164 in the soluble butelase-1 by LC-MS/MS sequencing. (A) Scheme of Asp-to-Snn conversion by dehydration. (B) MS/MS fragmentation spectrum of one tryptic digested peptide fragment containing Asp164.



Fig. S8 Examples of MALDI-TOF MS profiles of GN14-HV cyclization mediated by nBu1 and rBu1. The relative yield of cyclic products [%Cyc] were calculated by the ratio of peak areas as Area (cGN14)/Area (cGN14 + GN14-HV). The sodium and potassium ion adducts were also counted.



Fig. S9 Butelase-1 activity after freeze-thaw cycles. The enzyme solutions were either extraction buffer (without protectant) or enzyme storage buffer (with 20% sucrose). Activities of treated enzymes were examined by cyclization of GN14-HV.

A

>His6-TEV-GV-T1.2-NHV

MHHHHHHGSAENLYFQGVASRANDAPIVLLHGFTGWGRDEMVGFKYWGGVRGDIEQWLNDNGYRTYT LAVGPLSSNWDRACEAYAQLVGGTVDYGAAHAAKHGHARFGRTYPGLLPELKRGGLIHIIAHSQGGQ TARLLVSLLENGSQEEQEYAKAHNVSLSPLFEGGHRFVLSVTTIATPHDGTTLVNMVDFTDRFFDLQ KAVLKAAAVASNVPYTDSVYDFKLDQWGLRRQPGESFDHYVERLKRSPVWTSTDTARYDLSIPGAEA LNRWVQASPHTYYLSFSTERTERGAWTGNHYPELGMNAFSAVVCAPFLGSYRNPALGVDDRWLENDG IVNTVSMDGPKRGSSDRIVPYDGTIRKGVWNDMGTYNVDHLEVIGVDPNPLFPIRSFYLRLAEQLAS LDP<u>GSGSGSNHV</u>



Fig. S10 Confirmation of lipase cyclization (A) The sequence of linear precursor His6-TEV-GV-T1,2-NHV. (B) LC-MS of linear T1.2-NHV (calc. MW 43881, obs. MW 43897 (+16 Da, oxidation)) and circular lipase T1.2 (calc. MW 43627, obs. MW 43643 (+16 Da, oxidation)).

>ML-His6-Phytase-NHV

<u>MLVSHHHHHH</u>KHKLSDPYHFTVNAAAETEPVDTAGDAADDPAIWLDPKTPQNSKLITTNKKSGLVVYSLD GKMLHSYNTGKLNNVDIRYDFPLNGKKVDIAAASNRSEGKNTIEIYAIDGKNGTLQSMTDPDHPIATAIN EVYGFTLYHSQKTGKYYAMVTGKEGEFEQYELKADKNGYISGKKVRAFKMNSQTEGMAADDEYGRLYIAE EDEAIWKFSAEPDGGSNGTVIDRADGRHLTRDIEGLTIYYAADGKGYLMASSQGNSSYAIYDRQGKNKYV ADFRITDGPETDGTSDTDGIDVLGFGLGPEYPFGIFVAQDGENIDHGQKANQNFKIVPWERIADQIGFRP LANEQVDPRKLTDRSGK<u>NHV</u>



Fig. S11 Confirmation of phytase cyclization. (A) The sequence of linear precursor ML-His6-Phytase-NHV. (B) The tryptic-digested fragment of circular phytase containing the cyclization site. (C) The Asn-Met ligation site has been identified by MS/MS using MALDI-TOF mass spectrometry.

А



Fig. S12. Thermal shift assay of linear and circular lipase T1.2 and phytase PhyC.