Preparation and Purification of Active Recombinant Human Pancreatic Lipase in *Escherichia coli*

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[Abstract] Human pancreatic lipase (HPL) is the main lipolytic enzyme involved in the digestion of dietary fat. An active recombinant human pancreatic lipase (recHPL) was successfully prepared for the first time in an *Escherichia coli* (*E. coli*) expression system using a short Strep-tag II (ST II). The recHPL-ST II was solubilized with 8 M urea from the *E. coli* lysate and purified on a Strep-Tactin-Sepharose column. After refolding by stepwise dialyses against decreasing concentrations of urea in the presence of glycerol and Ca²⁺ for two days followed by gel filtration FPLC, 1.8-6 mg of active recHPL-ST II was obtained from 1 L of culture. Here we report the expression, purification, and optimized refolding procedures for active recHPL from *E. coli*, thus establishing it as a suitable system for the production of recHPL of high purity and scaling up.

Keywords: Human pancreatic lipase, *Escherichia coli*, Lipolytic activity, Refolding, Strep-tag II, Gel filtration

[Background] Mammalian lipase, essential for the dietary fat digestion and absorption, can be used for development of an anti-obesity treatment. However, obtaining pure and stable native mammalian lipase has been a challenge because it is often degraded by co-existing proteases in the pancreatic juice. Due to the limited availability of human tissue samples and their potential risk for disease transmission, it may be best to prepare HPL using recombinant expression systems. Several methods have been reported to express recHPL using yeast (Yang and Lowe, 1998), insect (Thirstrup *et al.*, 1993), or mammalian cells (Canalias *et al.*, 1994), which require culturing for at least 5, 6, or 10 days, respectively. The recHPLs obtained using these cells require strict quality control to achieve homogeneity because they are differentially glycosylated. The use of *E. coli* as an expression system can avoid these problems in addition to providing other benefits such as ease of culture and purification. Therefore, we have established a protocol for optimized expression, purification, and refolding of active recHPL from *E. coli*. This method requires only two days of culturing, is easy to scale up at low cost, and is convenient because it circumvents the need to control the glycosylation pattern of the recombinant protein. Although the recHPL is non-glycosylated and native porcine pancreatic lipase (PPL) is glycosylated, both lipases show lipolytic activity equal to native PPL against tributyrin and triolein as substrates (Kawaguchi *et al.*, 2018). It may be useful as an alternative to the PPL that has been used in the development of pharmaceutical products. For example, pancreatic enzyme replacement therapy for pancreatic exocrine

insufficiency (Lowe and Whitcomb, 2015), and screening of inhibitor compounds to provide an important avenue for anti-obesity therapy (Seyedan *et al.*, 2015). Other promising applications of recHPL are industrial biocatalysts for biotechnological processes (Sharma *et al.*, 2001).

Materials and Reagents

1. 50 ml tubes (Labcon, catalog number: 3181-345-008)
2. 15 ml tubes (Labcon, catalog number: 3131-345-008)
3. 1.5 ml tubes (BIO-BIK, catalog number: ST-0150F)
4. 0.2 ml PCR tubes with Dome Cap (Axygen, Funakoshi, catalog number: PCR-02D-C)
5. Pipette tips 10 μ l (BIO-BIK, catalog number: 1050N), 200 μ l (BIO-BIK, catalog number: PT-200), 1,000 μ l (BIO-BIK, catalog number: 804C)
6. 96-well microplates (Greiner Bio-One, catalog number: 655801)
7. Disposable 2 ml polystyrene columns (Pierce, catalog number: 29920)
8. Ultrafiltration discs, 10 kDa NMW (Millipore, catalog number: PBGC02510)
9. Cellulose acetate membrane filter, 0.45 μ m (Advantec, catalog number: C045A090C)
10. GL chromatodisc 0.45 μ m (GL Science, 25N)
11. PVDF membrane (Millipore, catalog number: IPVH00010)
12. Dialysis membrane (As One, catalog number: 2-316-02)
13. HiPrep 16/60 Sephacryl S-200 HR (GE Healthcare, catalog number: 17-1166-01)
14. Sterile Petri dishes (As One, catalog number: 1-7484-01)
15. *E. coli* DH5 α competent cells (Takara Bio, catalog number: 9057)
16. Stirred Ultrafiltration cells (Millipore, catalog number: 1532701)
17. Primers (Invitrogen)
 - Primer 1 (forward), 5'-AAAGAAGTTTGCTACGAAAGACTC-3'
 - Primer 2 (reverse), 5'-GATTGTGCCACACTCCCACTCG-3'
 - Primer 3 (forward), 5'-ACCAAAAGATGTCAACACCCGCTTCC-3'
 - Primer 4 (reverse), 5'-GACCAAGGCAATATATGGAGGGGTC-3'
 - Primer 5 (forward), 5'-ATGGTAGGTC TCAAATGAAAGAAGTTTGCTACGAAAGACTCG-3'
 - Primer 6 (reverse), 5'-ATGGTAGGTCTCAGCGCTACACGGTGTGAGGGTGAGCAG-3'
 - Primer 7 (forward), 5'-CCCAGCAGAACCTTGCTTTCAGG-3'
 - Primer 8 (reverse), 5'-CTTCCCAGATTCCGTCTATGTCC-3'
18. pASK-IBA3plus plasmid (IBA, catalog number: 2-1402-000)
19. Strep-Tactin-Sepharose (IBA, catalog number: 2-1201-010)
20. Human MTC Panel I (Clontech Laboratories, catalog number: 636742)
21. PfuTurbo DNA Polymerase (Agilent Technologies, catalog number: 600250)
22. KOD-Plus-Mutagenesis Kit (Toyobo Co., Ltd., catalog number: SMK-101)
23. MgSO₄·7H₂O (Wako, catalog number: 138-00415)
24. Anti-lipase antibodies (Abcam, catalog number: ab96100)

25. HRP-conjugated goat anti-rabbit antibodies (Kirkegaard and Perry Laboratories, catalog number: 074-15-061)
26. BSA, Albumin from bovine serum, Low salt (Wako, 017-15124)
27. Bsal (BioLabs, catalog number: R0535)
28. PCR purification kit (Wizard SV® Gel and PCR Clean-Up system) (Promega, catalog number: A9281)
29. Ligase enzyme T4 DNA Ligase (Toyobo Co. Ltd, Osaka)
30. BigDye Terminator™ Purification Kit (Thermo Fisher Scientific, catalog number: 4376484)
31. FastDigest® Eco31I (Thermo Fisher Scientific, catalog number: FDO293)
32. 10x FastDigest® Green Buffer (Thermo Fisher Scientific, catalog number: B72)
33. Ligation high ver.2 (TOYOBO, catalog number: LGK-201)
34. Ampicillin sodium (Wako, catalog number: 016-10373)
35. Bacto Agar (Becton, Dickinson and Co., catalog number: 214010)
36. Luria Bertani (LB) Broth Base (Invitrogen, catalog number: 12780-052)
37. Anhydrotetracycline (IBA, catalog number: 2-0401-002)
38. Urea (Wako, catalog number: 217-00171)
39. Dithiothreitol (DTT) (Wako, catalog number: 049-08972)
40. Desthiobiotin (Sigma-Aldrich, catalog number: D1411)
41. Ethylenediaminetetraacetic acid (EDTA) (Wako, catalog number: 345-01865)
42. Sodium chloride (NaCl) (Kanto Chemical Co., Inc., catalog number: 37144-86)
43. Sodium hydroxide (NaOH) (Wako, catalog number: 198-13765)
44. Phenylmethylsulfonyl fluoride (PMSF) (Nacalai Tesque, catalog number: 27327-94)
45. Tris (hydroxymethyl) aminomethane (Wako, catalog number: 204-07881)
46. Triton X-100 (Sigma-Aldrich, catalog number: T9284)
47. L-Arginine hydrochloride (Wako, catalog number: 018-04625)
48. CBB R-250 (Wako, catalog number: 031-17922)
49. Methanol (Wako, catalog number: 25183-70)
50. Acetic acid (Wako, catalog number: 017-00251)
51. L-Cystine dihydrochloride (Wako, catalog number: 03405322)
52. L-Cysteine (Wako, catalog number: 039-20652)
53. Glycerol (Wako, catalog number: 075-00611)
54. CaCl₂ (Wako, catalog number: 031-00435)
55. Tributyrin (Wako, catalog number: 207-02392)
56. Colipase, Pig (Bio-Rad, catalog number: 2148-1004)
57. Sodium taurodeoxycholate
58. LB agar plates (see Recipes)
59. LB medium (see Recipes)
60. Buffer A (see Recipes)
61. Lysis buffer (see Recipes)

62. Buffer A* (see Recipes)
63. Equivalent buffer (see Recipes)
64. Elution buffer (see Recipes)
65. Refolding buffer (see Recipes)
66. Running buffer (see Recipes)
67. Reaction buffer (see Recipes)

Equipment

1. 300 ml shaking flask (Iwaki, catalog number: 4070FK300)
2. 500 ml glass beaker (e.g., As One, catalog number: 2-5091-06)
3. Pipettes (Eppendorf, Reference 10-100 μ l, 0.5-10 μ l, catalog numbers: 4920000059, 4920000024)
4. Magnetic stirring bar (NALGENE, catalog number: 93-5521-5)
5. -80 °C freezer (Nihon Freezer MY BIO, model: VT-208)
6. Zymoreactor II thermal cycler (ATTO)
7. Prism 3100 Avant sequencer (Applied Biosystems)
8. Shaking incubator (e.g., Thomas, model: AT-12R)
9. Refrigerated bench-top centrifuge (e.g., Eppendorf, model: 5417R)
10. High speed centrifuge (Hitachi, model: CR20GIII)
11. Sonicator (Branson, model: Advanced Sonifier 250)
12. Spectrophotometer (DeNovix, model: DS-11)
13. Microplate spectrophotometer (DS Pharma Biomedical, model: Viento XS)
14. Fraction collector (e.g., Advantec, model: SF-2100)
15. Amicon™, Bioseparations Stirred Cell (Merck, catalog number: 8010)
16. AKTA Purifier chromatography system (GE Healthcare)
17. pH-stat AT-510 (Kyoto Electronics Manufacturing Co, Ltd.)

Software

1. UNICORN (GE Healthcare), control software for the AKTA purifier chromatography system
2. Gen 5 (Bio Tek), data collection and analysis software for the microplate reader
3. AT-Win (Kyoto Electronics Manufacturing Co, Ltd.), the control software for the pH-stat, automatic potentiometric titrator, AT-510

Procedure

This procedure to prepare recHPL includes five steps: (A) construction of expression plasmid, (B) expression in *E. coli*, (C) isolation, (D) refolding, and (E) purification.

A. Construction of HPL expression plasmid

ST II-fused rechHPL was cloned into the pASK-IBA3plus plasmid to allow expression in *E. coli*. The construct contains a C-terminal Strep-tag II to facilitate separation of rechHPL (Figure 1).

A

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P S N G Q M I N S * F L L T L Y H * * S Y F T T P Y Q * * R K V K * I V R Q K S
1 CCATCGAATG GCCAGATGAT TAATTCTAA TTTTGTGGA CACTATATCA TTGATAGAGT TATTTTACCA CTCCTATCA GTGATAGAGA AAAGTGAAAT GAATAGTTCG ACAAATATCT 120

R N N F V * L * E G D I Q M K E V C Y E R L G C F S D D S P W S G I T E R P L H
121 AGAAATAATT TTGTTTAACT TTAAGAAGGA GATATACAAA tgaagaagt tgcctacgaa agactcggct gcttcagtaga tgactccca ttacgaaag accctccat 240

I L P W S P K D V N T R F L L Y T N E N P N N F Q E V A A D S S S I S G S N F K
241 atattgctt ggtctccaaa agatgtcaac accogcttcc tccatatac taatgagaac ccaacaact ttcaagaagt tgcgcagat tcaatcagca tcaagtgtct caatttcaaa 360

T N R K T R F I I H G F I D K G E E N W L A N V C K N L F K V E S V N C I C V D
361 acaaatagaa aaactcgtt tattattcat ggattcatag acaagggaag agaaaactgg atggccaatg ttgcaagaa tctgttcaag gtgaaagtgt tgaactgtat ctgtgtggac 480

W K G G S R T G Y T Q A S Q N I R I V G A E V A Y F V E F L Q S A F G Y S P S N
481 tggaaagtgt gctccgaac tggatacaca caagcctcgc agaactcag gatcgtggga gcagaagtgt catatttgt tgaatttctt cagtcggcgt tccgttactc accctccaa 600

V H V I G H S L G A H A A G E A G R R T N G T I G R I T G L D P A E P C F Q G T
601 gtgcattgaa ttggccaacg cctgggtgct caagcgtcgt ggaaggcgtg aaggagaacc atggggacca ttggacgcat cacagggtgt gaccagcag aacctgtctt tcagggcaca 720

P E L V R L D P S D A K F V D V I H T D G A P I V P N L G F G M S Q V V G H L D
721 cctgaattag tccgattgga cccagcgtat gcaaaattgt tggatgtaat tcaacggatg ggtgcacca tagtcccaaa ttgggggttt ggaatgagcc aagtcgtggg ccacttagat 840

F F P N G G V E M P G C K K N I L S Q I V D I D G I W E G T R D F A A C N H L R
841 ttctttccaa atgggaagt ggaatgctt ggatgtaaaa agaacattct ctctcagatt gtggacatag acggaatctg ggaagggtct cgaactttgt cggcctgtaa tcaactaaga 960

S Y K Y Y T D S I V N P D G F A G F P C A S Y N V F T A N K C F P C P S G G C P
961 agctacaaat attacatgta tagcatcgtc aaactcgtgt gctttcgtgt attccctgtt gctcttaca aogtcttcc tgcaaaacag tgtttccctt gtcgaagtgt aggtcgtcca 1080

Q M G H Y A D R Y P G K T N D V G Q K F Y L D T G D A S N F A R W R Y K V S V T
1081 cagatgggtc actatgctga tagatatcct gggaacaaa atgatgtgg ccagaatttt tatctagaca ctggtgatgc cagtaatttt gcaagttgga ggtataaggt atctgtcaca 1200

L S G K K V T G H I L V S L F G N K G N S K Q Y E I F K G T L K P D S T H S N E
1201 ctgtctggaa aaaaggttac aggcacata ctagtcttct tgttcggaaa taaaggaaac tctaagcagt atgaattttt caagggcact ctcaaacag atagtactca ttccaatgaa 1320

F D S D V D V G D L Q M V K F I W Y N N V I N P T L P R V G A S K I I V E T N V
1321 ttgactcag atgtggatgt tggggactgt cagatgggta aatttattgt gtatacaat gtgatcaacc caactttacc tagagtggga gcatacaga ttatgtgga gacaaatgtt 1440

G K Q F N F C S P E T V R E E V L L T L T P C S A W S H P O F E K * * A * P V K
1441 ggaaaacagt tcaactctgt tagtcagaa accgtcagg aggaagttct gctcaccctc acacgtgtg GCGTTGGAG CCACCCGAG TTCCGAAAT AATAAGCTTG ACCTGTGAG 1560

* K M A H I V R H F F C L P F T A T A S R I S T R P V A A H * A R R V W W L R A
1561 TGAAAAATGG CGCATTGT CGCATTGT TTTGTCTGC CGTTACCGC TACTGCGTCA CGATCTCCA CGCGCCCTGT AGCGCGCAT TAAGCGCGC GGTGTGGTG GTTACGCGCA 1680

A * P L H L P A P * R P L L S L S S L P F S P R S P A F P V K L * I G G S L * G
1681 GCGTGACCGC TACACTGCC AGCGCCTAG CGCGCGTCC TTTCCTTCT TTCTGCCAC GTTCGCGGC TTTCCTCCGTC AAGCTCTAAA TCGGGGGCT CTTTAGGTT 1800

S D L V L Y G T S T P K N L I R V M V H V V G H R P D R R F F A L * R W S P R S
1801 TCCGATTAG TGCTTTACG CACCTGACC CCAAAAACT TGATTAGGTG GATGGTTCAC GTAGTGGGCC ATCGCCCTGA TAGACGGTT TTCCGCTTT GACGTGGAG TCCACGTCT 1920

L I V D S C S K L E Q H S T L S R S I L N V F Q * * A L L K F C Y V A R Y Y P V
1921 TTAATAGTGG ACTCTTGT CAACTGGAA CAACTCAA CCGTATCTG GTCATTCTT AACGTTTTC AATGATGAGC ACTTTTAAAG TTCTGCTATG TGGCGCGTA TTATCCGTA 2040

L T P G K S N S V A A Y T I L R M T W L S T H Q S Q K S I L R M A * Q * E N Y A
2041 TTGACCGCG GCAAGAGCAA CTCGGTCCG GCATACATA TTCTCAGAA GACTTGGTG AGTACTCAC AGTCACAGAA AAGCATCTTA CGGATGGCAT GACAGTAAGA GAATTATGCA 2160
    
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B

Figure 1. Sequence of rechHPL. A. Part of the nucleotide sequence of an HPL-pASK-IBA3plus plasmid and the deduced amino acid sequence. The entire sequence of HPL was confirmed using an autosequencer. Boxed region: the coding sequence of *HPL*, wavy underlines: the sequence of the linker, dotted underlines: the sequence of the ST II-tag. B. Structure of pASK-

IBA 3Plus-HPL plasmid.

1. Following the PCR protocol detailed below, amplify the coding sequence of HPL using the human pancreas cDNA library, Human MTC Panel I as the template for PCR using a Zymoreactor II (ATTO) thermal cyclers with following conditions:

[Primers]

Primer 1 (forward), 5'-AAAGAAGTTTGCTACGAAAGACTC-3'

Primer 2 (reverse), 5'-GATTGTGCCACACTCCCACTCG-3'

[Reaction mixture]

Component	Volume (μl)
Human MTC Panel I (pancreas)	2.5
10 μM Primer 1 (forward)	1.0
10 μM Primer 2 (reverse)	1.0
PfuTurbo DNA polymerase	0.5
10 mM dNTP	0.5
10x Cloned Pfu Reaction Buffer	2.5
ddH ₂ O	17.0
Total	25.0

[PCR conditions]

30 cycles of	95 °C	60 s (Denaturation)
	55 °C	60 s (Annealing)
	72 °C	60 s (Extension)

2. Separate the amplicon by agarose gel electrophoresis using 1% agarose gel.
3. Cut out the DNA band (expected size 1,350 bp) on the gel and purify with Wizard® SV Gel and PCR Clean-Up System to obtain insert DNA.
4. Perform a second PCR to replace the Eco311 restriction endonuclease recognition sequence GGTCTC contained in the insert DNA with GGTCAC using a Zymoreactor II (ATTO) thermal cyclers with following conditions.

[Primers]

Primer 3 (forward), 5'-ACCAAAAGATGTCAACACCCGCTTCC-3'

Primer 4 (reverse), 5'-GACCAAGGCAATATATGGAGGGGTC-3'

[Reaction mixture]

Component	Volume (μl)
50 ng/μl insert DNA	1.0
10 μM Primer 3 (forward)	1.5
10 μM Primer 4 (reverse)	1.5
25 mM MgSO ₄	2.0
2 mM dNTPs	5.0
10x PCR Buffer for KOD-Plus	5.0
KOD-Plus	1.0
ddH ₂ O	33.0
Total	50.0

[PCR conditions]

10 cycles of	94 °C	120 s
	98 °C	10 s
	68 °C	360 s

5. Separate the amplicon by agarose gel electrophoresis using 1% agarose gel.
6. Cut out the DNA band (expected size 1,350 bp) on the gel and purify it with Wizard® SV Gel and PCR Clean-Up System to obtain insert DNA.
7. Perform a third PCR to obtain insert DNA with Eco31 I restriction endonuclease recognition site added at both ends using Zymoreactor II (ATTO) thermal cycler under the following condition:

[Primers]

Primer 5 (forward), 5'-ATGGTAGGTC TCAAATGAAAGAAGTTTGCTACGAAAGACTCG-3'

Primer 6 (reverse), 5'-ATGGTAGGTCTCAGCGCTACACGGTGTGAGGGTGAGCAG-3'

[Reaction mixture]

Component	Volume (μl)
50 ng/μl insert DNA	1.0
10 μM Primer 3 (forward)	1.5
10 μM Primer 4 (reverse)	1.5
25 mM MgSO ₄	2.0
2 mM dNTPs	5.0
10x PCR Buffer for KOD-Plus	5.0
KOD-Plus	1.0
ddH ₂ O	33.0
Total	50.0

[PCR conditions]

Initial denaturation	94 °C	120 s
25 cycles of	94 °C	15 s
	65 °C	30 s
	68 °C	60 s

8. Purify the sequencing reactions using a BigDye Terminator™ Purification Kit.
9. Digest the amplicon 2 µl by adding FastDigest® Eco31I 1 µl, 10x FastDigest Green Buffer 2 µl, and ddH₂O 15 µl at 37 °C for 5 min.
10. Digest pASK-IBA3Plus 2 µl by adding FastDigest® Eco31I 1 µl, 10x FastDigest Green Buffer 2 µl, and ddH₂O 15 µl at 37 °C for 5 min.
11. Separate the digested samples by agarose gel electrophoresis using 1% agarose gel.
12. Cut out the DNA bands (expected sizes of insert DNA and pASK-IBA3Plus are 1,350 bp and 3,247 bp, respectively) on the gel and purify with Wizard® SV Gel and PCR Clean-Up.
13. Ligate the digested insert DNA with the digested pASK-IBA3plus to obtain an HPL-pASK-IBA3plus plasmid by DNA Ligation Kit, Ligation high Ver.2.
14. Sequence all constructed plasmids using the Prism 3100 Avant sequencer to confirm the sequences of the designed recombinant HPLs. Use the following primers:
Primer 7 (forward), 5'-CCCAGCAGAACCTTGCTTTCAGG-3'
Primer 8 (reverse), 5'-CTTCCCAGATTCCGTCTATGTCC-3'

B. Expression of rechHPL in *E. coli*

1. Transform 5 µl (10-fold dilution) of HPL-pASK-IBA3plus plasmid (*ca.* 2.5 ng) into 50 µl of *E. coli* DH5α competent cells in a 1.5 ml tube.
2. Incubate for 30 min on ice.
3. Incubate at 42 °C for 45 s.
4. Add 900 µl of LB medium and culture at 37 °C for 1 h.
5. Plate 100 µl of transformants on LB agar plates including 100 µg/ml ampicillin at 37 °C for 18 h.
6. Pick up a single colony and grow the transformants in 5 ml LB medium including 100 µg/ml ampicillin in a 50 ml tube at 37 °C for 18 h.
7. Transfer the bacterial suspension (3 ml) into 300 ml fresh LB medium including 100 µg/ml ampicillin in a shaking flask and culture for 3 h at 37 °C at 150 rpm.
8. Add 30 µl of 2 mg/ml anhydrotetracycline at a final concentration of 200 µg/L when the absorbance of the culture measured at 550 nm by a spectrophotometer (DeNovix) has reached 0.5 to induce expression of recombinant protein.
9. Incubate for 5 h at 30 °C with shaking at 120 rpm.
10. Centrifuge at 6,100 x *g* for 15 min at 4 °C using a high speed centrifuge (Hitachi) to harvest cells.
11. Discard the supernatant, resuspend the bacterial pellet in 15 ml of buffer A, and freeze at -80 °C in a 50 ml tube until use.

C. Isolation of rechPL

1. Thaw the frozen bacterial pellet.
2. Lyse the bacterial cell pellet by sonication for 2 min (30 s x 4 times, output: 4, duty cycle: 70%) on ice.
3. Centrifuge at 4,400 x *g* for 15 min at 4 °C using a high speed centrifuge (Hitachi).
4. Discard the supernatant and resuspend the pellet in 15 ml of lysis buffer.
5. Centrifuge at 28,000 x *g* for 10 min at 4 °C.
6. Discard the supernatant and wash the pellet twice with 30 ml of buffer A.
7. Centrifuge at 28,000 x *g* for 10 min at 4 °C.
8. Discard the supernatant and solubilize the washed inclusion bodies with 10 ml of buffer A* with agitation for 30 min at 4 °C.
9. Add 50 µl of 2 M DTT at a final concentration of 10 mM to the supernatant.
10. Incubate for 1 h at 37 °C with agitation at 100 rpm.
11. Dilute the solubilized proteins with 10 ml of buffer A*.
12. Dialyze against 150 ml of the equivalent buffer using the dialysis membrane (pore diameter = 50 Å) at 4 °C for 2 h to remove urea in a glass beaker with gentle stirring using a magnetic stirrer.
13. Centrifuge the dialyzed solution at 28,000 x *g* for 10 min at 4 °C.
14. Aspirate the supernatant and transfer it to a 50 ml tube.
15. Add 20 ml of ice-cold equivalent buffer to the 2 ml of Strep-Tactin-Sepharose in a polystyrene column using a Pasteur pipette.
16. Remove the equilibrated Strep-Tactin-Sepharose and add to the dialyzed protein solution in the 50 ml tube.
17. Incubate at 4 °C for 30 min with gentle shaking at around 30 rpm.
18. Pack the suspension mixture of Strep-Tactin-Sepharose into a polystyrene column.
19. Wash with 20 ml of equivalent buffer at a flow rate of 3 drops/s, collecting 70-drop fractions using a fraction collector at 4 °C (Figure 2).
20. Elute ST II-fused rechPL with elution buffer at a flow rate of 10 drops/s, collecting 22-drop fractions using a fraction collector at 4 °C.
21. Measure the absorbance of 200 µl of fractions using 96-well microplates, a microplate spectrophotometer (DS Pharma Biomedical), and software Gen 5 at 280 nm at room temperature.
22. Combine fractions with high A₂₈₀ readings corresponding to the eluted protein and store the eluted peak fractions in a 15 ml tube at -80 °C.

Figure 2. Separation of recHPL by Strep-Tactin-Sepharose column. A. Affinity chromatography profile on a Strep-Tactin-Sepharose column (Schmidt *et al.*, 1996). Elution was started from the fraction indicated by the arrow, and the peak was obtained at elution volume 19-26 ml. Column volume: 1.9 ml (*i.e.*, 7 mm x 50 mm), flow rate: 10 s/drop, temperature: 4 °C. B. SDS-PAGE and Western blot analysis of the recHPL-ST II. From left lane, “CBB” shows a CBB-stained 12% polyacrylamide gel. “Anti-HPL” shows an electroblotted PVDF membrane blocked with 3% BSA and stained using the primary antibody, anti-lipase antibodies (1:3,000) at room temperature for 1 h and the secondary antibody, HRP-conjugated goat anti-rabbit antibodies (1:5,000) at room temperature for 1 h followed by developed using ECL reagent (GE Healthcare). 1, Marker proteins; 2, solubilized protein from inclusion body; 3 and 4, recHPL-ST II purified on the Strep-Tactin-Sepharose column.

D. Refolding of RecHPL (Figure 3)

Figure 3. Procedure for refolding of isolated recHPL

1. Thaw the frozen eluted peak fractions and measure the absorbance at 280 nm at room temperature.
2. Add urea to a final concentration of 8 M to 7-12 ml of the purified 0.1-0.2 mg/ml recHPL in peak fractions.
3. Add 2 M DTT to a final concentration of 10 mM.
4. Incubate for 2 h at 37 °C with agitation at approximately 100 rpm.
5. Dialyze against 300 ml of refolding buffer containing a step-wise decrease in urea concentration (6 M and 4 M) at 4 °C for 2 h.
6. Dialyze against 300 ml of refolding buffer containing 2 M urea at 4 °C overnight.
7. Dialyze against 300 ml of refolding buffer containing step-wise decrease in urea concentration (1 M and 0.5 M) at 4 °C for 2 h.
8. Dialyze against 300 ml of refolding buffer containing 0 M urea at 4 °C for 1 h.
9. Centrifuge at 21,000 x g for 5 min and collect the supernatant to remove protein aggregates at 4 °C.

E. Purification of recHPL (Figure 4)

Note: Store all reagents and carry out all procedures at 4 °C.

1. Add the supernatant of the refolded recHPL to an Amicon™ Bioseparations Stirred Cell with an ultrafiltration disc (10 kDa NMWL).
2. Concentrate recHPL by 2-10 times using the stirred cell.
3. Filter the concentrated recHPL through the GL chromatodisc 0.45 µm (GL Science, 25N)
4. Filter the running buffer for FPLC through a cellulose acetate membrane filter, 0.45 µm.
5. Inject 1 ml of refolded recHPL into a HiPrep 16/60 Sephacryl S-200 HR column (column volume: 120 ml), pre-equilibrated with running buffer on an AKTA Purifier FPLC system at a flow rate of 0.5 ml/min.
6. Elute the column with 180 ml of running buffer at a flow rate of 0.5 ml/min, collecting 2 ml fractions.
7. Measure the absorbance of fractions at 280 nm and store approximately 5 fractions (each 2 ml) of the second peak as active recHPL.
8. Determine the lipolytic activity of recHPL using tributyrin as substrate by automated titration of fatty acids released with 10 mM NaOH using the pH-stat AT-510 (Kawaguchi *et al.*, 2018).

Figure 4. Purification of active recHPL by gel-filtration FPLC. Elution profile of recHPL on size exclusion. RecHPL after refolding was loaded onto a HiPrep 16/60 Sephacryl S-200 column (column volume: 120 ml) and eluted using 10 mM TBS containing 400 mM L-Arg at a flow rate of 0.5 ml/min at 4 °C. Protein peaks were detected by measuring absorbance at 280 nm using a UPC-900 monitor and AKTA Purifier. Active recHPL-ST II (1.8-6 mg) was obtained from 1 L of culture, and it showed a specific activity of 1.2-1.9 kU/mg. Specific activity of the fractions was measured using 0.11 M tributyrin emulsified in 1 ml reaction buffer as the substrate. The measurements were performed containing colipase in excess, 0.5 µg/50 µl. After addition of each fraction eluted by gel filtration FPLC in Figure 4 (0.5 µg/50 µl), the NaOH consumption was recorded at a reaction temperature of 37 °C using the automatic potentiometric titrator, pH-stat AT-510 (Kyoto Electronics Manufacturing Co. Ltd.). One unit was defined as the amount of enzyme that released 1.0 µmol of free fatty acid per min.

Data analysis

Protein expression is verified by Coomassie staining and Western blotting using anti-lipase antibodies (Abcam, ab96100). Chromatograms were recorded using UNICORN software. Gen 5 software was used for data collection and analysis for the microplate reader. The pH-stat AT-510 was controlled by AT-Win software.

Notes

1. Prepare 0.2 M PMSF stock solution in ethanol and dilute to buffer just before use.
2. In a comparison of TBS supplemented with 8 M urea, 1% Triton X-100, or 1% SDS, TBS containing 8 M urea is the most efficient method of solubilizing recHPL-ST II from *E. coli* lysates.

3. A Strep-Tactin-Sepharose column is an open column using gravity to move the solvent. The flow rate is controlled by the hydrostatic pressure on top of the column and the stopcock at the bottom of the column.
4. All the processes for isolation, dialysis for refolding, and purification of recHPL should be performed at 4 °C except for the denaturation processes with 8 M urea and 10 mM DTT, which must be done at 37 °C.
5. The addition of L-Arg showed a remarkable effect on the refolding of recHPL-ST II, resulting in a high yield, which must be attributable to the inhibition of aggregation of recHPL-ST II by L-Arg.

Recipes

1. LB agar plates
20 g/L LB Broth Base
15 g/L bacto agar
Autoclave at 121 °C, 2.6 bar for 20 min to sterilize
Add ampicillin (100 µg/ml) and pour the medium into sterile Petri dishes
2. LB medium
20 g/L LB Broth Base
Autoclave at 121 °C, 2.6 bar for 20 min to sterilize
3. Buffer A
10 mM Tris-HCl (pH 8.0)
150 mM NaCl
1 mM EDTA
4. Lysis buffer
10 mM Tris-HCl (pH 8.0)
150 mM NaCl
1 mM EDTA
0.5% Triton X-100
5. Buffer A*
10 mM Tris-HCl (pH 8.0)
150 mM NaCl
1 mM EDTA
8 M urea
1 mM PMSF
6. Equivalent buffer
10 mM Tris-HCl (pH 8.0)
150 mM NaCl
400 mM L-Arg
1 mM EDTA

- 1 mM PMSF
- 7. Elution buffer
 - 10 mM Tris-HCl (pH 8.0)
 - 150 mM NaCl
 - 400 mM L-Arg
 - 1 mM EDTA
 - 1 mM PMSF
 - 2.5 mM desthiobiotin
- 8. Refolding buffer
 - 10 mM Tris-HCl (pH 8.0)
 - 150 mM NaCl
 - 10% glycerol
 - 2.5 mM CaCl₂
 - 1 mM PMSF
 - 1 mM cysteine
 - 0.1 mM cystine
 - 400 mM L-Arg
 - Varying urea concentration: 6 M, 4 M, 2 M, 1 M, 0.5 M, and 0 M urea
- 9. Running buffer
 - 10 mM Tris-HCl (pH 8.0)
 - 150 mM NaCl
 - 400 mM L-Arg
- 10. Reaction buffer
 - 1 mM Tris-HCl (pH 7.5)
 - 4 mM sodium taurodeoxycholate
 - 100 mM NaCl
 - 5 mM CaCl₂

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Competing interests

The authors declare there are no conflicts of interest.

References

1. Canalias, F., Visvikis, A., Thioudellet, C. and Siest, G. (1994). [Stable expression of enzymatically active human pancreatic lipase in V79 cells: purification and characterization of the recombinant enzyme](#). *Clin Chem* 40(7 Pt 1): 1251-1257.
2. Kawaguchi, N., Date, K., Suzuki, Y., Tomita, C., Naradate, R., Higami, T., Nakamura, K., Aikawa, K. and Ogawa, H. (2018). [A novel protocol for the preparation of active recombinant human pancreatic lipase from *Escherichia coli*](#). *J Biochem* 164(6): 407-414.
3. Lowe, M. E. and Whitcomb, D. C. (2015). [Next generation of pancreatic enzyme replacement therapy: Recombinant microbial enzymes and finding the perfect lipase](#). *Gastroenterology* 149(7): 1678-1681.
4. Schmidt, G., Koepke, J., Frank, R. and Skerra, A. (1996). [Molecular interaction between the Strep-tag affinity peptide and its cognate target, streptavidin](#). *J Mol Biol* 255: 753-766.
5. Seyedan, A., Alshawsh, M. A., Alshagga, M. A., Koosha, S. and Mohamed, Z. (2015). [Medicinal plants and their inhibitory activities against pancreatic lipase: A review](#). *Evid Based Complement Alternat Med* 2015: 973143.
6. Sharma, R., Chisti, Y. and Banerjee, C. (2001). [Production, purification, characterization, and applications of lipases](#). *Biotechnol Adv* 19: 627-662.
7. Thirstrup, K., Carriere, F., Hjorth, S., Rasmussen, P. B., Woldike, H., Nielsen, P. F. and Thim, L. (1993). [One-step purification and characterization of human pancreatic lipase expressed in insect cells](#). *FEBS Lett* 327(1): 79-84.
8. Yang, Y. and Lowe, M. E. (1998). [Human pancreatic triglyceride lipase expressed in yeast cells: purification and characterization](#). *Protein Expr Purif* 13(1): 36-40.