Purification of a Protein Exhibiting Isoleucine 2-epimerase Activity from *Lactobacillus otakiensis* JCM 15040

Yuta Mutaguchi[1] and Toshihisa Ohshima[2*]

[1]Department of Biotechnology, Akita Prefectural University, Akita City, Japan; [2]Department of Biomedical Engineering, Osaka Institute of Technology, Osaka, Japan
[*]For correspondence: ohshima@bme.oit.ac.jp

[Abstract] Prominent accumulation of D-leucine, D-*allo*-isoleucine and D-valine was observed in the culture medium of the heterofermentative bacterial species, *Lactobacillus otakiensis* (*L. otakiensis*) JCM 15040. The racemase enzyme that resulted in this accumulation, isoleucine 2-epimerase, was purified from the bacterial cells. This is the first reported observation of such production of D-branched chain amino acids in lactic acid bacteria, and the first example of a racemase with isoleucine 2-epimerase activity in any organisms. In the described protocol, we introduce methods for purification of this protein from *L. otakiensis* JCM 15040. Because no specific ligand that has high affinity for this enzyme has been identified, the purification was performed using ammonium sulfate fraction, four types of column chromatography and preparative Native-PAGE, not using an affinity column chromatography. We hope that the protocol will provide useful information for purification of an enzyme that cannot easily be purified using an affinity column chromatography.

Materials and Reagents

1. 1.5 ml tube (ASONE Corporation, catalog number: 2-1998-02)
2. Dialysis membrane tube (molecular weight cutoff: 14,000) (EIDIA, catalog number: UC27-32-100)
3. Amicon Ultra 15 ml centrifugal filter 3 K device (Merck Millipore Corporation, catalog number: UFC500396)
4. Disposable homogenizer (1.5 ml scale) “Biomasher II” (NIPPI Corporation, catalog number: 320102)
5. Feeding tube (TERUMO CORPORATION, catalog number: SF-ET1725)
6. Syringe (10 ml scale) (TERUMO CORPORATION, catalog number: SS-10SZ)
7. Plug silicon (ASONE Corporation, catalog number: 6-336-03)
8. Needle (TERUMO CORPORATION, catalog number: NN-2238R)
9. Purification of the isoleucine 2-epimerase
    a. *L. otakiensis* JCM 15040 obtained from Japan Collection of Microorganisms (JCM)
    b. TOYOPEARL Phenyl-650M column
    Note: Pack 50 ml of TOYOPEARL Phenyl-650M resin in a chromatography column (diameter: 2.5 cm, length: 10 cm) (Tosoh Bioscience LLC, catalog number:

14478).

c. TOYOPEARL Butyl-650M column

Note: Pack 50 ml of TOYOPEARL Butyl-650M resin in a chromatography column (diameter: 2.5 cm, length: 10 cm) (Tosoh Bioscience LLC, catalog number: 07477).

d. TOYOPEARL SuperQ-650M column

Note: Pack 50 ml of TOYOPEARL SuperQ-650M resin in a chromatography column (diameter: 2.5 cm, length: 10 cm) (Tosoh Corporation, catalog number: 17227).

e. Acrylamide (Wako Pure Chemical Industries, Siyaku, catalog number: 016-00765)

f. *N, N'*-Methylenebisacrylamide (Nacalai Tesque, catalog number: 22402-02)

g. Ammonium persulfate (Wako Pure Chemical Industries, Siyaku, catalog number: 7727-54-0)

h. *N, N, N', N'*-Tetramethylethylenediamine (Nacalai Tesque, catalog number: 33401-72)

i. Ammonium sulfate (Wako Pure Chemical Industries, Siyaku, catalog number: 019-03435)

j. Sodium chloride (Wako Pure Chemical Industries, Siyaku, catalog number: 198-01675)

k. Lactobacilli MRS Broth (MRS medium powder) (BD, catalog number: 288130) (see Recipes)

l. 50 mM sodium phosphate buffer (pH 7.2) containing 1 mM EDTA and 1 mM dithiothreitol (see Recipes)

Note: Unless otherwise indicated, this buffer is used as the standard buffer throughout the purification procedures.

i. Sodium dihydrogenphosphate dihydrate (Wako Pure Chemical Industries, Siyaku, catalog number: 192-02815)

ii. Disodium hydrogenphosphate 12-water (Wako Pure Chemical Industries, Siyaku, catalog number: 196-02835)

iii. Ethylenediamine-*N, N, N', N'*-tetraacetic acid, disodium salt, dehydrate (EDTA) (Dojindo Molecular Technologies, catalog number: N001)

iv. Dithiothreitol (Nacalai Tesque, catalog number: 14128-04)

m. Red Sepharose CL-4B column (Ohshima and Sakuraba, 1986) (see Recipes)

Note: According to Recipes, prepare Red Sepharose CL-4B resin by attaching Reactive Red 120 (Sigma-Aldrich, catalog number: R0378-50G) to Sepharose® CL-4B (Sigma-Aldrich, catalog number: CL4B200-100ML). Pack 10 ml of Red Sepharose CL-4B resin in a chromatography column (diameter: 1.5 cm, length: 12 cm).

n. 1.5 M Tris-HCl buffer (pH 8.8) (see Recipes)

i. 2-Amino-2-hydroxymethyl-1, 3-propanediol (Tris base) (Wako Pure Chemical Industries, Siyaku, catalog number: 207-06275)

ii. Hydrochloric acid (Nacalai Tesque, catalog number: 18402-45)

o. 0.5 M Tris-HCl buffer (pH 6.8) (see Recipes)
   i. 2-Amino-2-hydroxymethyl-1, 3-propanediol (Tris base)
   ii. Hydrochloric acid

p. Native-PAGE electrophoresis buffer (see Recipes)
   i. 2-Amino-2-hydroxymethyl-1, 3-propanediol (Tris base)
   ii. Glycine (Wako Pure Chemical Industries, Siyaku, catalog number: 077-00735)

q. 2x Native-PAGE gel loading buffer (see Recipes)
   i. 2-Mercaptoethanol (Nacalai Tesque, catalog number: 21418-42)
   ii. Sucrose (Wako Pure Chemical Industries, Siyaku, catalog number: 193-00025)
   iii. Bromophenol blue (KANTO CHEMICAL, catalog number: 04319-30)

10. Isoleucine 2-epimerase activity assay
    a. Pyridoxal-5'-phosphate (Nacalai Tesque, catalog number: 29606-74)
    b. L-isoleucine (PEPTIDE INSTITUTE, catalog number: 2712)
    c. Flavin adenine dinucleotide disodium salt (Nacalai Tesque, catalog number: 16010-06)
    d. 4-Aminoantipyrine (Nacalai Tesque, catalog number: 01907-52)
    e. Phenol (Wako Pure Chemical Industries, Siyaku, catalog number: 168-12721)
    f. D-Amino acid oxidase from porcine kidney (Sigma-Aldrich, catalog number: A5222-200UN)
    g. Peroxidase (TOYOBO BIO CHEMICAL DEPT, catalog number: PE0-301)
    h. 0.5 M sodium phosphate buffer (pH 8.0) (see Recipes)
       i. Sodium dihydrogenphosphate dihydrate
       ii. Disodium hydrogenphosphate 12-water

Equipment

A. Purification of the isoleucine 2-epimerase
   1. Incubator (TITEC CORPORATION, model: BR-43FM)
   2. Centrifuge (Hitachi Koki Co., model number: CR 21F and TOMY SEIKO CO., model number: MX-300)
   3. Multi-Beads shocker (Yasui Kikai Corporation)
   4. Electrophoresis apparatus
   5. Column chromatography equipment (see Representative data)
      a. Magnetic stirrer (ASONE Corporation, model: HS-50E)
      b. Two-way stopcock (Bio-Rad Laboratories, catalog number: 7328102)
      c. Three-way stopcock (TERUMO CORPORATION, catalog number: TS-TR1K)
      d. Chromatography column (diameter: 2.5 cm, length: 10 cm) (Bio-Rad Laboratories, catalog number: 7372512)
      e. Chromatography column (diameter: 1.5 cm, length: 12 cm) (Bio-Rad Laboratories,

catalog number: 7321010)

f. Fraction tube (15 ml scale) (ASONE Corporation, catalog number: 2-8007-02)

B. Isoleucine 2-epimerase activity assay

1. Spectrophotometer (Shimadzu Scientific Instruments, model number: UVmini-1240)
2. Water bath (TOKYO RIKAKIKAI CO., model number: NTT-20S)
3. Vortex (TAITEC CORPORATION, catalog number: 0061271-000)

Procedure

A. Purification of the isoleucine 2-epimerase

1. Prepare 5 test tubes including 10 ml of MRS medium for preculture.
2. Inoculate a part of a colony of *L. otakiensis* JCM 15040 into each MRS medium (10 ml) in the test tube. The preculture is performed statically at 30 °C for 36 h.
3. The main culture is started by addition of the preculture media (10 ml x 5 tubes = 50 ml) into 10 L of MRS medium.
4. *L. otakiensis* JCM 15040 is cultivated statically at 30 °C for 30 h in an incubator, after which cells are pelleted by centrifugation (8,000 *x g* for 30 min at 4 °C). The cell pellet (*ca.* 32.2 g, wet weight) is used as the starting material for purification of protein exhibiting isoleucine 2-epimerase activity. Unless otherwise indicated, all purification procedures are carried out at room temperature, and the enzyme solution is stored at 4 °C.
5. To prepare a crude extract, the cells are washed twice with about four volumes (120 ml) of the standard buffer, and suspend in 120 ml of the same buffer. On the occasion of the washing, the cells are collected by centrifugation (8,000 *x g* for 30 min at 4 °C). Next, they are disrupted using a Multi-Beads Shocker (2,500 rpm for 60 sec at 2 °C, 5 times), and centrifuged (10,000 *x g* for 30 min at 4 °C). The resultant supernatant (100 ml) is used as the crude extract.
6. The crude extract (100 ml) is mixed with two volumes of 3.6 M $(NH_4)_2SO_4$ dissolved in the standard buffer. After incubation on a magnetic stirrer for 4 h at 4 °C, the mixture is centrifuged (10,000 *x g* for 30 min at 4 °C), and the supernatant (280 ml) is retrieved.
7. The collected supernatant (280 ml) is applied to a TOYOPEARL Phenyl-650M column. This column chromatography separates proteins on the basis of hydrophobic interactions between the proteins and the resin. Before the supernatant is loaded, pre-equilibrate the column with 10 column volumes (500 ml) of 2.4 M $(NH_4)_2SO_4$ dissolved in the standard buffer. After the loading, the column is washed once with three column volumes (150 ml) of the same buffer and proteins are eluted using a linear gradient of 2.4 to 0.4 M $(NH_4)_2SO_4$ in the buffer [used buffer: 250 ml of the buffer with 2.4 M $(NH_4)_2SO_4$ and 250 ml of the buffer with 0.4 M $(NH_4)_2SO_4$]. The elution is performed at a flow rate of about 1 ml/min, and 50 fractions including about 10 ml of

elute are collected.

8. Assay the enzyme activity in each fraction (see section B “Isoleucine 2-epimerase activity assay” below), and choose five active fractions showing higher activity than other fractions. The five active factions (about 50 ml) are mixed, and then dialyzed against 100 volumes (5 L) of the standard buffer at 4 °C. After 4 h, the standard buffer (5 L) is changed and dialysis is continuously performed at 4 °C for 12 h.
9. This first dialysate (60 ml) is mixed with two volumes of 3.6 M $(NH_4)_2SO_4$ dissolved in the standard buffer, and the mixture is applied to a TOYOPEARL Butyl-650M column. This column chromatography separates proteins on the basis of hydrophobic interactions between the proteins and the resin. Before the supernatant is loaded, pre-equilibrate the column with 10 column volumes (500 ml) of 2.4 M $(NH_4)_2SO_4$ dissolved in the standard buffer. After the loading, the column is washed once with three column volumes (150 ml) of the same buffer, and proteins are eluted using a linear gradient of 2.4 to 0.4 M $(NH_4)_2SO_4$ in the buffer [used buffer: 250 ml of the buffer with 2.4 M $(NH_4)_2SO_4$ and 250 ml of the buffer with 0.4 M $(NH_4)_2SO_4$]. The elution is performed at a flow rate of about 1 ml/min, and 50 fractions including about 10 ml of elute are collected. The five active fractions are pooled and dialyzed as described above.
10. This second dialysate (60 ml) is applied to a TOYOPEARL SuperQ-650M column. This column chromatography separates proteins on the basis of ionic interactions between the proteins and the resin. Before the supernatant is loaded, pre-equilibrate the column with 10 column volumes (500 ml) of the standard buffer. After the loading, the column is washed once with three column volumes (150 ml) of the buffer, and proteins are eluted using a linear gradient of 0 to 250 mM NaCl in the buffer (used buffer: 250 ml of the standard buffer and 250 ml of the buffer with 250 mM NaCl). The elution is performed at a flow rate of about 1 ml/min, and 50 fractions including about 10 ml of elute are collected. The five active fractions are pooled and dialyzed as described above.
11. SDS-PAGE of this third dialysate shows that this dialysate includes not only isoleucine 2-epimerase, but also a putative NAD^+-dependent alcohol dehydrogenase (data not shown). To remove this NAD^+-dependent dehydrogenase, the third dialysate (50 ml) is applied to a Red Sepharose CL-4B column. Because the red dye immobilized in this column binds to a wide variety of NAD^+- and $NADP^+$-dependent enzymes but not isoleucine 2-epimerase, the NAD^+-dependent enzyme and isoleucine 2-epimerase are separated by pooling the flow-through of this column chromatography. Before the supernatant is loaded, pre-equilibrate the column with 10 column volumes (100 ml) of the standard buffer. After the loading, the column is washed the column once with three column volumes (30 ml) of the standard buffer. During sample loading and column washing, the flow-through (about 80 ml) as the active fractions is pooled. The resultant enzyme solution is then concentrated to 300 µl using an Amicon Ultra

centrifugal filter 3 K device, and then the concentrated enzyme solution is stored at 4 °C.

12. Native-polyacrylamide gel electrophoresis (Native-PAGE) of the concentrated enzyme solution (300 µl) is performed on a polyacrylamide slab gel for further enzyme purification; 30 µl of the enzyme solution is loaded on each of 10 lanes. The electrophoresis is performed using the method of Laemmli (Laemmli, 1970) with some modifications; buffers without sodium dodecyl sulfate are used, and the protein sample is not heated during the pretreatment procedures. After electrophoresis using a constant current of 20 mA for 90 min, the gel is cut into 16 pieces using a cutter and a ruler. The gel pieces are individually crushed in 300 µl of the standard buffer using Biomasher II, and the resultant solutions are centrifuged (17,000 *x g* for 15 min at 4 °C) (Figure 2). The enzyme activity of the supernatants is assayed and the active enzyme solution is used as the final purified enzyme solution from *L. otakiensis* JCM 15040.

B. Isoleucine 2-epimerase activity assay

1. Prepare the first step reaction mixture in 1.5 ml tube. The composition of the first step reaction mixture is shown in the following table. Run this reaction for 1 h at 30 °C. The 1.5 ml tube containing the reaction mixture is incubated in a water bath.

The first step reaction mixture (500 µl)

0.5 M sodium phosphate buffer (pH 8.0)	100 µl
0.5 mM pyridoxal-5'-phosphate	100 µl
50 mM L-isoleucine	100 µl
Enzyme solution	50 µl
H_2O	150 µl

2. Boil the first step reaction mixture in 1.5 ml tube for 10 min, and thus cool it to room temperature.
3. Prepare the second step reaction mixture by adding reagents shown in the following table into the first step reaction mixture. Run this reaction for 15 min at 37 °C. The 1.5 ml tube containing the reaction mixture is incubated in a water bath.

The second step reaction mixture (1,000 µl)	
The first step reaction mixture	500 µl
0.5 M sodium phosphate buffer (pH 8.0)	100 µl
0.4 mM fravin-adenine dinucleotide	50 µl
4 mM 4-aminoantipyrine	50 µl
40 mM phenol	50 µl
5 Unit/ml D-amino acid oxidase	50 µl
20 Unit/ml horseradish peroxidase	50 µl
H_2O	50 µl

4. Apply the second step reaction mixture to a spectrophotometer, and thus measure absorbance at 500 nm. A measurement of the reaction mixture without the enzyme solution is used as a blank data.

Representative data

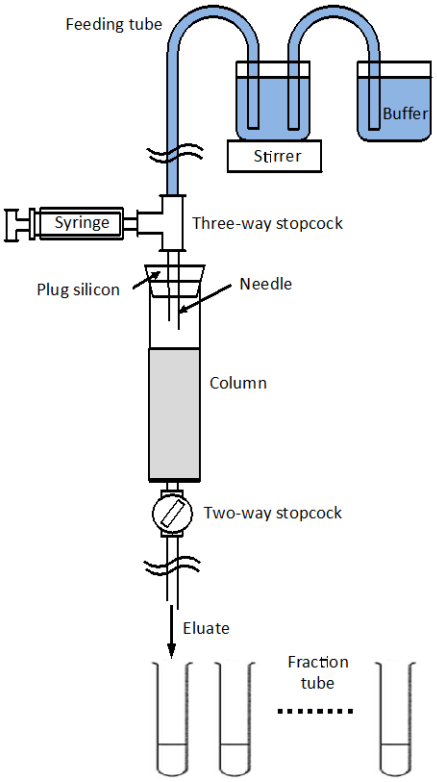

Figure 1. Image of the column chromatography equipment

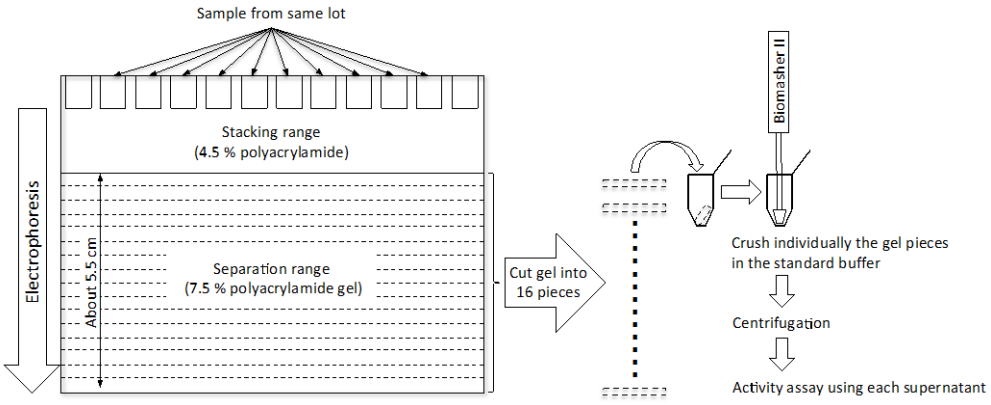

Figure 2. Image of the purification procedure using Native-PAGE

Notes

Isoleucine 2-epimerase activity assay

For choosing active fraction, the enzyme activity is rapidly assayed by the following 2 step reactions (Figure 3). At first step, isoleucine 2-epimerase reaction with L-isoleucine as a substrate produces D-*allo*-isoleucine. At next step, D-amino acid oxidase-peroxidase coupling reaction is performed. In this coupling reaction, D-amino acid oxidase reaction produces H_2O_2 in oxidization of D-*allo*-isoleucine, and thus peroxidase reaction produces indophenol compound (λmax: 500 nm, ε: 6.39 $mM^{-1}{\cdot}cm^{-1}$) from 4-aminoantipyrine and phenol using oxidation power of H_2O_2. The isoleucine 2-epimerase activity is indirectly assayed by the increase in absorbance at 500 nm occurring from the indophenol compound production.

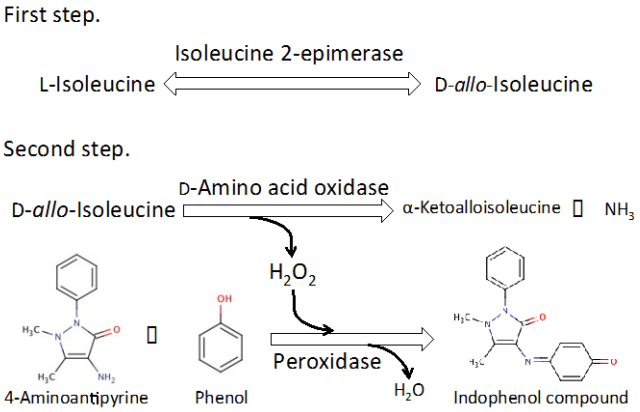

Figure 3. Principle of the assay method for isoleucine 2-epimerase activity

Recipes

A. Purification of the isoleucine 2-epimerase

1. MRS medium (1 L)
   Mix 55 g of MRS medium powder with 800 ml of dH_2O
   Add dH_2O to 1,000 ml
   Autoclave
   Stored at room temperature
2. 50 mM sodium phosphate buffer (pH 7.2) containing 1 mM EDTA and 1 mM dithiothreitol (1 L)
   Mix 3.90 g of $NaH_2PO_4{\cdot}2H_2O$ and 8.95 g of $Na_2HPO_4{\cdot}12H_2O$ with 800 ml of dH_2O
   Add 2 ml of 0.5 M EDTA (pH 8.0)
   pH to 7.2 with HCl or NaOH (aq)
   Add dH_2O to 1,000 ml
   Autoclave
   Add and mix 0.154 g of dithiothreitol
   Stored at 4 °C
3. Red Sepharose CL-4B
   Add Reactive Red 120 (1 g in 100 ml of dH_2O) and NaCl (22%, 20 ml) into slurry of Sepharose 4B (100 ml) previously washed with dH_2O
   Tumble the mixture at 25 °C for 30 min slowly
   Add 2 g of Na_2CO_3 to the mixture
   Coupling is achieved by shaking gently for about 4 days at 25 °C
   Wash the slurry with 2 L of dH_2O and 2 L of 1 M KCl
   Store the Red-Sepharose 4B at 4 °C
4. 1.5 M Tris-HCl buffer (pH 8.8, 100 ml)
   Mix 18.2 g of Tris base with 80 ml of dH_2O
   pH to 8.8 with HCl
   Add dH_2O to 100 ml
   Autoclave
   Store at room temperature
5. 0.5 M Tris-HCl buffer (pH 6.8, 100ml)
   Mix 6.1 g of Tris base with 80 ml of dH_2O
   pH to 6.8 with HCl
   Add dH_2O to 100 ml
   Autoclave
   Stored at room temperature
6. Polyacrylamide slab gel for Native-PAGE (thickness, 1 mm; wide, 106 mm; high, 80 mm)

Separate range (7.5% polyacrylamide gel, 6 ml)	
1.5 M Tris-HCl buffer (pH 8.8)	1.5 ml
29% (w/v) acrylamide + 1% (w/v) *N, N'*-methylene bis acrylamide solution	1.5 ml
10% (w/v) ammonium persulfate	200 µl
dH_2O	2.8 ml
N, N, N', N'-Tetramethylethylenediamine	3 µl

Stacking range (4.5% polyacrylamide gel, 3 ml)	
0.5 M Tris-HCl buffer (pH 6.8)	0.75 ml
29% (w/v) Acrylamide + 1% (w/v) *N', N'*-methylene bis acrylamide solution	0.45 ml
10% (w/v) Ammonium peroxodisulphate	100 µl
dH_2O	1.7 ml
N, N, N', N'-Tetramethylethylenediamine	3 µl

7. Native-PAGE electrophoresis buffer (1 L)
   Mix 3.03 g of Tris base, 14.4 g of glycine and 900 ml of dH_2O
   Add dH_2O to 1 L
   Autoclave
   Stored at room temperature
8. 2x Native-PAGE gel loading buffer (10 ml)
   Mix 2.5 ml of 0.5 M Tris-HCl buffer (pH 6.8), 1 ml of 2-mercaptoethanol, 1 g of sucrose, 1 mg of bromophenol blue and 8 ml of dH_2O
   Add dH_2O to 10 ml
   Stored at −20 °C

B. Isoleucine 2-epimerase activity assay
   1. 0.5 M sodium phosphate buffer (pH 8.0, 100 ml)
      Mix 1.06 g of $NaH_2PO_4{\cdot}2H_2O$ and 15.5 g of $Na_2HPO_4{\cdot}12H_2O$ with 80 ml of dH_2O
      pH to 8.0 with HCl or NaOH (aq)
      Add dH_2O to 100 ml
      Autoclave
      Stored at room temperature

Acknowledgments

In this method, we have modified Laemmli's method for Native-PAGE. In addition, this work was supported by a grant for Promotion of Basic Research Activities for Innovate Bioscience from the Bio-oriented Technology Research Advancement Institution (BRAIN) and JSPS KAKENHI Grant Number 2402734.

References

1. Ohshima, T. and Sakuraba, H. (1986). Purification and characterization of malate dehydrogenase from the phototrophic bacterium, *Rhodopseudomonas capsulata*. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology* 869(2): 171-177.
2. Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227(5259): 680-685.

