Published in final edited form as:

Methods Enzymol. 2017 ; 587: 97–113. doi:10.1016/bs.mie.2016.09.055.

Production of Human ATG Proteins for Lipidation Assays

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Abstract

Humans express several orthologs of yeast Atg8, in the LC3 and GABARAP families, which play crucial roles in autophagy through their covalent ligation to lipids, typically phosphatidylethanolamine (PE), in a process known as lipidation. Lipidation of LC3 and GABARAP regulates numerous facets of the autophagy process, including regulating expansion of the phagophore membrane, recruiting selected cargoes for degradation, and providing an autophagosome membrane-bound platform mediating dynamic interactions with other regulatory proteins. LC3 and GABARAP are families of related ubiquitin-like proteins (UBLs) (referred to here collectively as LC3/GABARAP), and their lipidation involves a divergent UBL conjugation cascade including ATG7, ATG3, and ATG12~ATG5-ATG16L1 acting as E1, E2, and E3 enzymes, respectively. ATG7 initiates LC3/GABARAP conjugation by catalyzing their C-terminal adenylation and conjugation to the catalytic cysteine of ATG3. Ultimately, the ATG12~ATG5-ATG16L1 complex catalyzes LC3/GABARAP ligation to a primary amino group on PE or other acceptor lipids. This chapter describes methods for expressing and purifying human LC3 or GABARAP, ATG7, ATG3, and the ATG12~ATG5-ATG16L1 complex for in vitro studies of LC3/GABARAP lipidation.

1. INTRODUCTION

In eukaryotic cells, autophagy is a tightly regulated process responsible for degrading and recycling cellular components, and eliminating toxic assemblies and pathogens by sequestering cargo within an autophagosome, which fuses with a lysosome (or vacuole in yeast) harboring hydrolytic enzymes (Klionsky & Emr, 2000; Kobayashi, 2015; Wileman, 2013). Several key aspects of this regulation involve ubiquitin-like proteins (UBLs) in the Atg8 family, which form LC3 and GABARAP subfamilies in higher eukaryotes. LC3/GABARAP proteins have molecular weights in solution of approximately 14–17 kDa, but their electrophoretic migration is relatively accelerated upon ligation to lipids such as in autophagic membranes. LC3/GABARAP ligation to lipids (lipidation) plays numerous roles

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in regulation, including influencing the formation, maturation, and size of autophagosomes, as well as recruiting selected cargoes and regulatory proteins (Ichimura et al., 2000; Ohsumi & Mizushima, 2004; Nakatogawa, Ichimura, & Ohsumi, 2007; Tanida, Ueno, & Kominami, 2008).

LC3 and GABARAP lipidation is orchestrated by a UBL ligation system, specifically conjugating the carboxyl group of the C-terminal glycine of LC3/GABARAP to the amine group of phosphatidylethanolamine (PE) (or potentially other lipids such as phosphatidyl serine) in autophagic membranes (Hanada et al., 2007; Ichimura et al., 2000; Klionsky & Emr, 2000; Sou, Tanida, Komatsu, Ueno, & Kominami, 2006; Tanida, Ueno, & Kominami, 2004a, 2004b). This involves a specialized enzyme cascade, where ATG7 acts as the E1 and ATG3 acting as the E2 to produce covalent, reactive thioester-linked ATG3~LC3/GABARAP (“~” denotes a covalent protein interaction and “-” denotes a noncovalent complex) intermediates. Efficient lipidation requires an ATG12~ATG5-ATG16L1 multiprotein complex acting as the E3. Notably, formation of this E3-like assembly first requires a related conjugation cascade, wherein the UBL ATG12 is covalently conjugated to ATG5 by ATG7 acting as the E1 and ATG10 acting as the E2 (Hanada et al., 2007; Noda, Fujioka, Hanada, Ohsumi, & Inagaki, 2013), and then the ATG12~ATG5 binds ATG16L1 to achieve full E3-like activity toward ATG3~LC3/GABARAP intermediates. Mutations or dysfunction of proteins in the LC3/GABARAP lipidation cascade results in severe diseases and poor prognosis in various eukaryotic organisms including humans (Cadwell, Patel, Komatsu, & Virgin, 2009; Dezelak, Repnik, Koder, Ferkolj, & Potocnik, 2016; Kim et al., 2016; Lenz, Vierstra, Nurnberger, & Gust, 2011; Moloughney et al., 2011; Reed, Morris, Owczarczyk, & Lukacs, 2015; Tindwa et al., 2015; Wu et al., 2014; Xi et al., 2016; Xue, Chiu, & Oleinick, 2010).

Given the importance of LC3 and GABARAP lipidation, and potential interest in targeting their conjugation for therapeutic purposes (Cheng, Ren, Hait, & Yang, 2013; Puri & Chandra, 2014), purified proteins for assaying LC3/GABARAP lipidation and performing structural analyses would be useful (Bae & Park, 2010; Kaiser et al., 2013; Metlagel, Otomo, Ohashi, Takaesu, & Otomo, 2014; Metlagel, Otomo, Takaesu, & Otomo, 2013; Nath et al., 2014; Otomo, Metlagel, Takaesu, & Otomo, 2013; Sakoh-Nakatogawa et al., 2013; Taherbhoy et al., 2011). However, our optimal expression and purification procedures differ between the human LC3/GABARAP lipidation components and their yeast counterparts (Qiu, Hofmann, Coats, Schulman, & Kaiser, 2013). Thus, in this chapter, we describe detailed methods for expression and purification of human ATG proteins involved in LC3/GABARAP lipidation, including ATG7, ATG3, ATG12~ATG5-ATG16L1, and a method for expressing LC3B as exemplary for the human Atg8 orthologs.

2. EXPRESSION OF HUMAN LC3B, ATG7, ATG3, ATG12~ATG5, AND ATG16L1

2.1 General Overview of Protein Expression and Purification

LC3B (emblematic of LC3/GABARAP family members), ATG3, and ATG16L1 were expressed as GST-fusion proteins in *Escherichia coli*, whereas ATG7 and the ATG12~ATG5

conjugate were expressed through distinct methods in insect cells (Berger, Fitzgerald, & Richmond, 2004). An overview of the subsequent purification strategies is shown in Fig. 1.

2.2 Expression Constructs

1. A truncated version of LC3B, corresponding to residues 1–120 that form the activated form with a C-terminal glycine that is the site of chemical reactions (Tanida et al., 2004a, 2004b), is expressed from pGEX-4T-1 (Table 1, Fig. 2A) in *E. coli*.
2. Full-length ATG7 was expressed from a modified version of pFastBac, with TEV-cleavable N-terminal GST tag (pFastBac-GST) (Table 1, Fig. 2B) for expression in insect cells.
3. Full-length ATG3 was expressed from a modified version of pGEX-4T-1 harboring a cleavage site for TEV protease instead of thrombin between GST and ATG3 (pGEX-TEV) (Table 1, Fig. 2C), for expression in *E. coli*.
4. A truncated version of ATG16L1 corresponding to residues 1–69 was expressed from pGEX-TEV (Table 1, Fig. 2D) in *E. coli*.
5. The ATG12~ATG5 subcomplex corresponds to a truncated version of ATG12 (residues 52–140) that comprises the ubiquitin-like domain (Hanada & Ohsumi, 2005) linked via an isopeptide bond between its C-terminus to Lys130 on full-length ATG5 (Hanada et al., 2007; Otomo et al., 2013). The linkage between ATG12 and ATG5 is achieved by their coexpression with ATG7 and ATG10 in insect cells using the biGbac system (Weissmann et al., 2016). First, four separate plasmids were generated. The coding sequences were inserted into pLIB vectors, with ATG7, and ATG10 untagged, ATG5 harboring a N-terminal TEV cleavable His-tag, and ATG12 (52–140) with a N-terminal TEV cleavable GST-tag (Table 1, Fig. 2E). Next, GST-TEV-ATG12 (52–140), His-TEV-ATG5, ATG10, and ATG7 along with the promoter and terminator from the pLIB vectors were sequentially amplified using primers described in Weissmann et al. (2016), and coassembled into the pBIG1a vector (Table 1, Fig. 2F) using the method of Gibson Assembly (Gibson, 2011; Gibson et al., 2009; Weissmann et al., 2016).
6. All constructs were generated through transformation into DH5alpha (NEB), bacterial growth, minipreps, and verified by automated sequencing. The recommended antibiotic concentrations are: 100 µg/mL for ampicillin, 50 µg/mL for kanamycin and spectinomycin, 25 µg/mL for chloramphenicol, 10 µg/mL for tetracyclin, and 7 µg/mL for gentamycin.

2.3 Protein Expression

2.3.1 Expression in *E. coli*

1. Transform plasmid into BL21-CodonPlus (DE3)-RIL *E. coli* competent cells (Agilent Technologies), plate on LB-agar with appropriate antibiotics. Incubate at 37 °C for 12–18 h.

2. Prepare a starter culture from a single colony in 5 mL LB medium with antibiotics. Shake culture at 37 °C, 200 rpm for 10–16 h.
3. Dilute the 5 mL starter culture into 1 L LB medium with appropriate antibiotics. Grow cells at 37 °C, 200 rpm, and monitor closely by measuring the absorbance at 600 nm. When OD₆₀₀ reaches 0.8–1.0, add IPTG at a final concentration of 0.6 mM, change shaker temperature to 18°C, and grow with shaking at 200 rpm for 16–20 h.

2.3.2 Expression in Insect Cells

1. Transform pFastBac or pBIG1a vector containing expression cassettes into DH10EMBacY *E. coli* competent cells (Geneva Biotech). This enables transposase-mediated recombination resulting in a bacmid containing expression cassettes (Bac-to-Bac Baculovirus Expression System, Thermo Fisher Scientific).
2. Screen for colonies harboring bacmids that have undergone recombination using the blue/white identification method described in the Bac-to-Bac Baculovirus Expression System manual. Briefly, a LB plate containing ampicillin, kanamycin, tetracycline, and gentamycin, is spread with 40 µL 40 mg/mL X-Gal (dissolved in dimethylformamide) and 40 µL 0.1 M IPTG is incubated at 37 °C for 4 h to let the dimethylformamide evaporate before plating the transformation product.
3. Incubate at 37 °C for at least 24 h until colonies grow and background colonies with a clear blue color appear. White colonies are candidates for harboring a correctly recombined bacmid. PCR screen for candidates that contains all the target genes. At the same time, restreak white colonies on a fresh plate with X-gal, IPTG, and the four antibiotics. Include a blue colony as a control for color development. Perform bacmid extraction from culture of a single white colony with positive PCR screening result.
4. All insect cell medium used in this chapter must be prewarmed at 27 °C. Transfect to obtain baculovirus in five steps. (a) Coat 6 well flat bottom culture plate (CoStar) with 5 X10⁶ Sf9 insect cells (Thermo Scientific) per well and incubate at 27 °C for 30 min for cell attachment. (b) Dilute 200 ng bacmid with 100 µL SFX serum-free insect cell medium (GE Healthcare Life Sciences) in a 15 mL polystyrene tube (Falcon) (do not use polypropylene tube). Mix 10 µL FuGENE (Promega) with diluted bacmid. After incubation at 27 °C for 30 min, add 900 µL medium to achieve a 1 mL volume. (c) Aspirate medium from Sf9 cells. Evenly apply 1 mL bacmid-FuGENE mixture onto cells and incubate for 4 h. (d) Add another 1 mL medium and incubate for 4 days. (e) Collect supernatant that contains P1 virus by syringe and pass through a 0.2 µm PES syringe filter (Thermo Fisher Scientific). Virus can be stored at 4 °C.
5. Amplify virus to generate a P2 stock: Plate 3 X10⁷ Sf9 cells on a 150 mm²⁵ mm dish (Corning) and incubate 30 min at 27 °C to allow cell adherence (add more SFX serum-free insect cell medium if liquid volume is below 12 mL or fails to

cover the whole dish surface). Apply 0.5 mL P1 virus dropwise to adhered cells, gently rock to spread virus evenly, and incubate plate at 27 °C for 2 h. Then add more medium to a total volume of 25 mL per dish and incubate at 27 °C for another 3 days. Collect supernatant that contains virus into a 50 mL conical tube followed by centrifugation at 500 X g for 10 min to remove floating cells. Save the clarified supernatant as P2 amplified virus.

6. Amplify virus to generate a P3 stock. Use 1 mL P2 virus for infection using the same procedure for P2 amplification, except may scale up depending on the ultimate culture size for protein production. The volume of P3 amplified virus produced by each 150 mm cell culture dish supports approximately 2 L Hi5 cells during infection to produce protein. Repeat if further virus amplification is needed.
7. Infection for protein production. Pellet Hi5 insect cell (Thermo Fisher Scientific) culture at log phase by centrifugation at 500 X g for 10 min. Then resuspend the cell pellet by medium to reach a cell density of 1.25×10^7 /mL in PC Erlenmeyer flasks (VWR). Add 5 mL P3 amplified virus per 100 mL resuspended cells, then incubate cells in shaker at 27 °C, 75 rpm for 2 h with ambient CO₂.
8. Diluted cell culture by adding medium to a cell density of 2.5×10^6 /mL (make sure the culture volume is less than 50% of the flask volume). Let cells grow and express target protein in shaker at 27 °C, 155 rpm for 3 more days with ambient CO₂.

3. PROTEIN PURIFICATION

3.1 Preparation of Cell Lysate

1. Harvest bacteria cells by centrifugation at 5900 X g for 12 min at 4 °C, or insect cells at 500 X g for 15 min at 4 °C.
2. Resuspend cell pellet in 15 mL lysis buffer per liter bacteria culture, or 50 mL per liter insect cell culture. Lysis buffer for all proteins except the ATG12~ATG5 subcomplex is: 25 mM Tris-HCl pH 7.6, 200 mM NaCl, 2.5 mM phenylmethanesulfonyl fluoride (PMSF), 5 mM 2-Mercaptoethanol (BME). Lysis buffer for ATG12~ATG5 complex is: 1 PBS, 400 mM NaCl, 2.5 mM PMSF, 5 mM BME. Also add 20 mg/L aprotinin, 10 mg/L leupeptin, or a protease inhibitor cocktail of choice to insect cell lysis buffer.
3. Lyse bacteria cells by sonicating 7 times, or insect cells 3 times, 10 s each time with intermittent cooling on ice.
4. Clear lysate by centrifugation at 32,000 X g for 1 h at 4 °C. Collect the supernatant and keep it on ice.

3.2 Glutathione-Affinity Chromatography for LC3B, ATG7, ATG3, and ATG12~ATG5-ATG16L1

For GST-thrombin-LC3B, GST-TEV-ATG7, and GST-TEV-ATG3, glutathione-affinity chromatography is directly applied to their corresponding cell lysates. To obtain GST-TEV-ATG12 (52–140)~HisTEV-ATG5-GST-TEV-ATG16L1 (1–69) complex, cell lysates from insect cells and bacteria are mixed at 1:1 volume ratio and incubated at 4 °C for 10 min before processing.

1. Wash Glutathione Sepharose 4B (GS4B, GE Healthcare Life Sciences) 3 times with 10 column volumes of wash buffer in a chromatography column at 4 °C. Alternatively, wash the beads with 3 volumes of wash buffer by centrifugation at 500 X g for 5 min at 4 °C, decanting the wash buffer, and repeating twice. Suggested bead volumes: 0.75 mL per 1 L bacterial culture, or 2 mL per 1 L Hi5 insect cell culture, which is appropriate for both GST and nickel beads and all species of ATG proteins described in this chapter. Wash buffer is the same as lysis buffer but without PMSF, aprotinin, leupeptin, or any other proteasome inhibitor.
2. Incubate lysate with washed beads. Gently rock the mixture at 4 °C for 2 h for GS4B beads or 1 h for nickel beads to allow full binding.
3. Transfer lysate with beads into a Poly-Prep chromatography column (Bio-Rad) at 4 °C. Allow lysate to flow through by gravity to generate an even resin surface in column. Although the flow-through is unneeded in principle, it is a good practice to save the flow-through for troubleshooting in the event of problems.
4. If the lysate-bead solution is too thick to pass through the column, centrifuge the solution in 50 mL tubes at 500 X g for 5 min at 4 °C. Collect the supernatant as flow through. Gently resuspend pelleted beads in 10 column volumes of wash buffer and follow step 3.
5. Wash resin in column with 10 column volumes of wash buffer without disturbing the flat resin surface. Repeat 3 times.

3.3 Protease-Mediated Elution From Beads for Purification of LC3B, ATG3, and ATG7

1. Measure rough protein concentration of bead turbid solution using BioRad protein assay with a standard curve. Bovine serum albumin is a suitable standard.
2. Store 10 µL of uncleaved sample as a negative control for cleavage efficiency check before adding protease. Add thrombin protease to LC3B (1 mg thrombin per 100 mg of total protein), or TEV protease to ATG7 and ATG3 (2 mg TEV per 100 mg total protein). Keep gently rotating on a rocking shaker overnight to allow full cleavage.
3. To be certain that all desired protein is cleaved from the GST-tag, examine the bead slurry by SDS-PAGE, using a 15% acrylamide gel and Coomassie-staining, comparing cleaved samples to uncleaved samples (LC3B: Fig. 3, Lanes a and b; ATG7: Fig. 4, Lanes a and b; ATG3: Fig. 5, Lanes a and b).

4. Transfer slurry into an empty Poly-Prep chromatography column pre-rinsed with wash buffer, and collect flow through. When liquid completely passes through, apply another 1 column volume of wash buffer on the top of resin, and collect this second fraction containing residual protein.

3.4 Glutathione-Mediated ATG12~ATG5-ATG16L1 Elution From GS4B

1. Gently apply 0.7 resin volume of GS4B elution buffer on the top of resin surface while keeping stopcock closed. After a 10-min incubation, open the stopcock and collect the eluate in a tube. Repeat 6 times and keep elution fractions in separate tubes. GS4B elution buffer: wash buffer supplemented with 20 mM glutathione (reduced, free acid) (EMD Millipore), adjust pH by NaOH solution to the same as wash buffer.
2. Run each eluted fraction on a 15% SDS-PAGE gel to determine which fractions contain the majority of target protein. Pool desired fractions.

3.5 Nickel-Affinity Chromatography, Imidazole-Mediated Elution From Nickel Beads, and TEV Cleavage to Obtain ATG12 (52–140)~ATG5-ATG16L1 (1–69) Complex

After glutathione chromatography and glutathione mediated elution, the excessive GST-TEV-ATG16L1 (1–69) is further removed by nickel affinity chromatography.

1. Wash HIS-Select Nickel Affinity Gel (Sigma-Aldrich) 3 times with 10 column volumes of wash buffer in a chromatography column at 4 °C. Then perform nickel-affinity chromatography to enrich the ATG5-bound complex. Apply the glutathione elution of GST-TEV-ATG12 (52–140)~His-TEV-ATG5-GST-TEV-ATG16L1 (1–69) to the HIS-Select Nickel Affinity Gel beads and incubate following the same procedure described above for glutathione-affinity chromatography.
2. Perform an imidazole elution following steps described in glutathione elution using nickel bead elution buffer (wash buffer supplemented with 100 mM imidazole pH 7.5).
3. Store 10 µL uncleaved sample as a negative control to check cleavage efficiency. Add TEV protease (2 mg TEV per 100 mg total protein) to imidazole eluate, then dialyze in wash buffer while performing cleavage overnight at 4 °C.
4. Examine cleavage completeness by SDS-PAGE, using a 15% acrylamide gel and Coomassie-staining (Fig. 6, Lanes a and b).
5. The cleaved GST and HisMBP tags are removed from ATG12 (52–140)~ATG5-ATG16L1 (1–69) complex by further cation ion exchange purification described below.

3.6 Ion Exchange Chromatography on ATG3 and ATG12 (52–140)~ATG5-ATG16L1 (1–69)

Perform anion ion exchange to tag-free ATG3 and cation ion exchange to tag-free ATG12 (52–140)~ATG5-ATG16L1 (1–69) accordingly to their isoelectric points, before ultimately purifying by gel filtration chromatography. This step is optional for LC3B and ATG7.

1. Equilibrate a 5 mL HiTrap SP (cation exchange) or a Q (anion exchange) HP column (GE Healthcare Life Sciences) on an AKTA FPLC (GE Healthcare Life Sciences) in 95% buffer A with 5% buffer B. Cation exchange buffer A: 25 mM HEPES pH 7.0, 5 mM DTT. Anion exchange buffer A: 25 mM Tris-HCl pH 8.0, 5 mM DTT. Buffer B corresponds to buffer A with 1 M NaCl.
2. Dilute protein samples with buffer A to a final NaCl concentration of 50 mM.
3. Clarify samples by centrifugation at 4000 X g for 5 min at 4 °C before loading onto a FPLC system. If loading is through a sample pump, equilibrate the sample pump in buffer A. The ATG proteins described in this chapter can be concentrated to 10 mg/mL if desired to reduce volume prior to loading onto FPLC.
4. Load sample onto column with a suggested loading rate of 2 mL/min. After loading, wash column by 5 column volumes of 95% buffer A with 5% buffer B.
5. After all unbound sample is washed out, initiate gradient, raising buffer B proportion from 5% to 50% over 25 column volumes at 2 mL/min flow rate, and collecting fractions with a volume of 2 mL.
6. Examine peak fractions by SDS-PAGE with Coomassie-stained 15% acrylamide gels to determine which fractions contain the majority of the ATG proteins (the combined fractions of ATG3 is shown in Fig. 5, Lane c; ATG12~ATG5-ATG16L1 is not shown).

3.7 Gel Filtration Chromatography for LC3B, ATG7, ATG3, and ATG12~ATG5-ATG16L1

Superdex 200 10/300 GL columns (GE Healthcare Life Sciences) are used for all the ATG proteins described here, except for LC3B using a Superdex 75 10/300 GL column (GE Healthcare Life Sciences). Protein samples of LC3B and ATG7 used in this step are directly collected from protease cleavage, while ATG3 and ATG12~ATG5-ATG16L1 are prepurified by ion exchange.

1. Equilibrate a Superdex 75/200 column on an AKTA FPLC system with storage buffer (25 mM Tris-HCl pH 7.6, 200 mM NaCl, 5 mM DTT). Increase NaCl concentration to 500 mM in the buffer used for LC3B, or to 300 mM in the buffer used for ATG12 (52~140)~ATG5-ATG16L1 (1~69).
2. Concentrate protein samples by Amicon Ultra Centrifugal Filter Units (EMD Millipore) with appropriate molecular weight cutoff. Transfer concentrated protein samples to one or multiple 1.7 mL microcentrifuge tubes. Perform centrifugation at 16,000 X g for 10 min at 4 °C to clarify samples before loading onto a FPLC system.
3. Load 1~2 mL clarified protein sample onto a Superdex 200 column, run gel filtration in storage buffer, and collect fractions with a fraction volume of 0.5 mL.
4. Examine peak fractions by SDS-PAGE with Coomassie-stained 15% acrylamide gels to determine which fractions contain the majority of the ATG proteins. Pool

desired fractions (the pooled fractions of LC3B, ATG7, ATG3, and ATG12~ATG5-ATG16L1 are shown in Fig. 3, Lane c; Fig. 4, Lane c; Fig. 5, Lane d; Fig. 6, Lane c, respectively).

4. STORING PURIFIED PROTEINS

Purified proteins can be concentrated to approximately 10 mg/mL and stored in 20 μ L aliquots at -80°C after flash-freezing in liquid nitrogen. Thaw on ice for use and refreeze for storage up to twice to ensure protein activity.

5. CONCLUDING REMARKS

Proteins prepared as described earlier are eligible for multiple biochemical assays, including LC3B lipidation. In our experience, aliquots of purified ATG proteins stored at -80°C are stable for up to a year, giving consistent results in assays for LC3B lipidation, formation of thioesterbonded ATG7~LC3B and ATG3~LC3B intermediates, binding using BioLayer Interferometry (Octet Red, ForteBio) or isothermal titration calorimetry (ITC), and for structural studies using NMR and crystallography.

Acknowledgments

This work was supported by ALSAC, the NIH R01GM077053, the Howard Hughes Medical Institute, and AHA 14POST19890021. B.A.S. is an Investigator of the Howard Hughes Medical Institute.

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Figure 3

Figure 4

Figure 5

Figure 6

Fig. 1.

Flow-chart indicating the expression system and purification strategies for production of human ATG proteins for lipidation assays.

Fig. 2.

Graphical representation of plasmids used in this chapter. (A) pGEX-4T-1-LC3B for LC3B (1–120) expression in *E. coli*. (B) pFastBac-GST-ATG7 to construct bacmid for ATG7 expression in insect cells. (C) pGEX-TEV-ATG3 for ATG3 expression in *E. coli*. (D) pGEX-TEV-ATG16L1 for ATG16L1 (1–69) expression in *E. coli*. (E) pLIB plasmid containing a GST-TEV-ATG12 (52–140), His-TEV-ATG5, ATG10, or ATG7 expression cassette to generate pBIG1a plasmid. (F) pBIG1a-ATG12-ATG5-ATG10-ATG7 plasmid to construct bacmid for ATG12 (52–140)~ATG5 expression in insect cells.

Fig. 3. 15% SDS-PAGE showing LC3B (1–120) at different steps of purification. Lane **M** shows the molecular weight markers. Lane **a** shows constituents of bead slurry after binding GS4B resin. Lane **b** shows constituents of bead slurry after thrombin treatment. Lane **c** shows final purified LC3B (1–120) after gel filtration chromatography.

Fig. 4. 15% SDS-PAGE showing ATG7 at different steps of purification. Lane **M** shows the molecular weight markers. Lane **a** shows constituents of bead slurry after binding GS4B resin. Lane **b** shows constituents of bead slurry after TEV treatment. Lane **c** shows final purified ATG7 after gel filtration chromatography.

Fig. 5. 15% SDS-PAGE showing ATG3 at different steps of purification. Lane **M** shows the molecular weight markers. Lane **a** shows constituents of bead slurry after binding GS4B resin. Lane **b** shows constituents of bead slurry after TEV treatment. Lane **c** shows purified ATG3 after anion ion exchange chromatography. Lane **d** shows final purified ATG3 after gel filtration chromatography.

Fig. 6. 15% SDS-PAGE showing ATG12 (52–140)~ATG5-ATG16L1 (1–69) complex at different stages of purification. Lane **M** shows the molecular weight markers. Lane **a** shows elution from nickel-affinity chromatography, capturing His- and His-MBP tags on ATG5 and ATG16L1 (1–69), respectively. This is the second step in the purification, following an initial purification by glutathione affinity chromatography to capture GST-tag on ATG12. Lane **b** shows product of TEV cleavage reaction preformed during dialysis. Lane **c** shows final purified ATG12 (52–140)~ATG5-ATG16L1 (1–69) complex after subsequent cation ion exchange and gel filtration chromatography.

Table 1

Plasmids Used in This Chapter

Proteins Expressed	Plasmids Generated	Functions
LC3B (1–120)	pGEX-4T-1-LC3B	Protein expression in E. coli
ATG7	pFastBac-GST-ATG7	To generate bacmid for protein expression in insect cells
ATG3	pGEX-TEV-ATG3	Protein expression in E. coli
ATG12 (52–140)~ATG5	pLIB-GST-TEV-ATG12	To generate pBIG1a plasmid
	pLIB-His-TEV-ATG5	To generate pBIG1a plasmid
	pLIB-ATG10	To generate pBIG1a plasmid
	pLIB-ATG7	To generate pBIG1a plasmid
	pBIG1a-ATG12-ATG5-ATG10-ATG7	To generate bacmid for protein expression in insect cells
ATG16L1 (1–69)	pGEX-TEV-ATG16L1	Protein expression in E. coli