

Chapter 3

Recombinant Collagen Trimers from Insect Cells and Yeast

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Summary

At least 28 proteins have now been defined as collagens (Trends Genet. 20:33–43, 2004; J. Biol. Chem. 281:3494–3504, 2006), but many of those recently discovered are present in tissues in such small amounts that their isolation for characterization at the protein level has so far been impossible. Some of the fibril-forming collagens are used as a biomaterial in numerous medical applications and as a delivery system for various drugs (3,4). The collagens used in all these applications have been isolated from animal tissues and are liable to cause allergic reactions in some subjects and carry a risk of disease-causing contaminants (3,4). An efficient recombinant expression system for collagens can thus be expected to have numerous scientific and medical applications. The systems commonly used for expressing other proteins in lower organisms are not suitable as such for the production of recombinant collagens, however, as bacteria and yeast have no prolyl 4-hydroxylase activity and insect cells have insufficient levels of it. Prolyl 4-hydroxylase, an $\alpha_2\beta_2$ tetramer in vertebrates, plays a central role in the synthesis of all collagens, as 4-hydroxyproline-deficient collagen polypeptide chains cannot form triple helices that are stable at 37°C (5,6). All attempts to assemble an active prolyl 4-hydroxylase tetramer from its subunits in vitro have been unsuccessful, but active recombinant human prolyl 4-hydroxylase has been produced in insect cells, yeast, and *Escherichia coli* by coexpression of its α - and β -subunits (7–9).

Key words: Collagen, Biomaterial, Prolyl 4-hydroxylase, Recombinant, Coexpression, Baculovirus, Insect cells, Yeast, *Pichia pastoris*.

1. Introduction

At least 28 proteins have now been defined as collagens (1, 2), but many of those recently discovered are present in tissues in such small amounts that their isolation for characterization at

the protein level has so far been impossible. Some of the fibril-forming collagens are used as a biomaterial in numerous medical applications and as a delivery system for various drugs (3, 4). The collagens used in all these applications have been isolated from animal tissues and are liable to cause allergic reactions in some subjects and carry a risk of disease-causing contaminants (3, 4). An efficient recombinant expression system for collagens can thus be expected to have numerous scientific and medical applications. The systems commonly used for expressing other proteins in lower organisms are not suitable as such for the production of recombinant collagens, however, as bacteria and yeast have no prolyl 4-hydroxylase activity and insect cells have insufficient levels of it. Prolyl 4-hydroxylase, an $\alpha_2\beta_2$ tetramer in vertebrates, plays a central role in the synthesis of all collagens, as 4-hydroxyproline-deficient collagen polypeptide chains cannot form triple helices that are stable at 37°C (5, 6). All attempts to assemble an active prolyl 4-hydroxylase tetramer from its subunits in vitro have been unsuccessful, but active recombinant human prolyl 4-hydroxylase has been produced in insect cells, yeast, and *Escherichia coli* by coexpression of its α - and β -subunits (7–9). We have developed efficient expression systems to produce recombinant human collagens with stable triple helices in insect and yeast cells by simultaneous coexpression with the recombinant human collagen prolyl 4-hydroxylase (8, 10–15). This chapter describes detailed procedures for the production of stable recombinant human type III collagen in insect cells and in the yeast *Pichia pastoris*. These methods can be applied to the expression of other recombinant collagen types, both homotrimeric and heterotrimeric.

2. Materials

2.1. Materials for Expression of Human Type III Procollagen in Insect Cells (10)

1. pVL1392 and p2Bac baculovirus expression vectors (Invitrogen, San Diego, CA).
2. Full-length cDNAs for the (16) and (17) subunits of human prolyl 4-hydroxylase and the pro1 chain of human type III procollagen [pro1(III)] (18).
3. Wizard Plus Maxiprep DNA purification system (Promega, Madison, WI).
4. Sf9 and High Five (H5) insect cells (Invitrogen).
5. TNM-FH medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum.
6. 6-well plates and 60- and 100-mm tissue culture Petri dishes.

7. BaculoGold DNA and Transfection Buffers A (Grace's Medium with 10% fetal bovine serum) and B (25 mM Hepes, pH 7.1, 125 mM CaCl₂, 140 mM NaCl) (Pharmingen, San Diego, CA).
8. 3% Seaplaque low-melting-point agarose (FMC, Rockland, ME) in H₂O (autoclaved).
9. L-Ascorbic acid phosphate (Wako, Osaka, Japan).
10. PBS (0.15 M NaCl, 20 mM phosphate, pH 7.4).
11. Homogenization buffer (300 mM NaCl, 0.2% Triton X-100, and 70 mM Tris-HCl, pH 7.4).
12. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) apparatus.
13. PIINP radioimmunoassay (Farmos Diagnostica, Turku, Finland).
14. Pepsin (Boehringer Mannheim, Mannheim, Germany), trypsin type XIII (Sigma), chymotrypsin type VII (Sigma), trypsin inhibitor type II-S (Sigma).

**2.2. Materials for
Expression of Human
Type III Procollagen
in the Yeast *Pichia
pastoris* (8)**

1. pAO815, pPIC9, and pPICZ B *P. pastoris* expression vectors (Invitrogen). pYM25 containing the *Saccharomyces cerevisiae* ARG4 selection marker (obtained from Dr. James Cregg, Oregon Graduate Institute of Science and Technology, Portland, OR).
2. *his4*, *arg4* *P. pastoris* host strain (obtained from Dr. James Cregg).
3. Yeast extract peptone dextrose medium (YPD: 1% yeast extract, 2% peptone, 2% dextrose), minimal dextrose medium [MD: 1.34% yeast nitrogen base with ammonium sulfate and without amino acids (YNB), $4 \times 10^{-5}\%$ biotin, 1% dextrose], buffered glycerol-complex medium (BMGY: 0.1% yeast extract, 0.2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, $4 \times 10^{-5}\%$ biotin, 1% glycerol), buffered minimal methanol medium (BMM: 100 mM potassium phosphate, pH 6.0, 1.34% YNB, $4 \times 10^{-5}\%$ biotin, 0.5% methanol). Recipes for various *Pichia* media can be found in Version 3.0 of the Invitrogen *Pichia* Expression Kit Manual (19).
4. Zeocin (Invitrogen).
5. Triple-baffled Erlenmeyer flasks and silicone sponge closures (Sigma).
6. Electroporation device and cuvettes.
7. Acid-washed glass beads (Sigma).
8. 1 M sorbitol.
9. 5% glycerol, 50 mM sodium phosphate buffer, pH 7.4.

3. Methods

3.1. Expression of Recombinant Human Type III Procollagen in Insect Cells

3.1.1. Generation of Recombinant Baculovirus Expression Vectors (10)

1. A double promoter baculovirus expression vector for recombinant human prolyl 4-hydroxylase (p2Bac4PH $\alpha\beta$) was generated by cloning the cDNAs for its α and β subunits into the *NotI* site downstream of the p10 promoter and the *BamHI* site downstream of the polyhedrin promoter of p2Bac, respectively (12). A *NotI* site was generated in the subunit cDNA 46 bp upstream of the translation initiation (ATG) codon by PCR (*see Note 1*).
2. A *BglII* site was created 16 bp upstream of the translation initiation codon to the full-length cDNA for the pro1 chain of human type III procollagen (*see Note 1*). The cDNA was digested with *BglII* and *XbaI* and the insert was ligated to *BglII*–*XbaI*-digested pVL1392, generating pVLRhproCIII.
3. The recombinant expression vectors p2Bac4PH $\alpha\beta$ and pVLRhproCIII were isolated using the Wizard Plus Maxiprep DNA purification system and sterilized with a 0.22- μ m syringe filter.

3.1.2. Generation of Recombinant Baculoviruses (20)

1. The recombinant baculoviruses 4PH $\alpha\beta$ and rhproCIII were generated by cotransfection with BaculoGold baculovirus DNA (*see Note 2*). 2×10^6 Sf9 cells were seeded on 60-mm tissue culture Petri dishes. Cells were allowed to attach for at least 30 min. The culture medium was then removed from the plates and 1 ml of Transfection Buffer A was added. BaculoGold DNA, 0.5 μ g, was mixed with 5 μ g of the baculovirus expression vector DNA and incubated for 5 min at room temperature. One milliliter of Transfection Buffer B was mixed well with the DNA mixture, and added dropwise to the 60-mm insect cell plate. The plate was gently rocked after the addition of every 3–5 drops of the transfection solution.
2. The plates were incubated at 27°C for 4 h, the transfection solutions were removed, and 4 ml of TNM-FH medium supplemented with 10% fetal bovine serum was added.
3. The plates were incubated at 27°C for 4 days. The medium containing the recombinant virus was collected and amplified once by infecting 6×10^6 Sf9 cells in a 100-mm Petri dish with 500 μ l of the collected transfection medium. The plates were incubated at 27°C for 3 days before the amplification medium was harvested.
4. The amplified recombinant viruses were plaque-purified (*see Note 3*). 2×10^6 Sf9 cells were seeded on 60-mm Petri dishes and allowed to attach for at least 30 min. Three millilitres of serial dilutions (10^{-4} , 10^{-5} , 10^{-6} , 10^{-7}) of the recombinant

viruses in TNM-FH supplemented with 10% fetal bovine serum were prepared. The medium from the 60-mm plates was removed and 1 ml of the virus dilutions was added on duplicate plates. The plates were incubated at 27°C for 1 h. Three percents of SeaPlaque agarose was melted in a microwave oven, cooled to 37°C, and two volumes of the culture medium preheated to 37°C was added to obtain a 1% agarose overlay. The virus dilutions were removed and 4 ml of the agarose overlay was added. The plates were incubated in a humid environment at 27°C for 6 days. Agar plugs containing single clear plaques were removed with a sterile pasteur pipette (*see Note 4*) into 1 ml of the culture medium and the virus particles were eluted by incubation at 4°C overnight.

5. The plaque-purified viruses were amplified three times for 3 days at 27°C (amplifications I, II, and III) to obtain high-titer virus stocks (*see Note 5*). After the first amplification test infections were performed to screen the viruses for the production of the recombinant proteins of interest (*see Subheading 3.1.3*). AI (amplification I): 1×10^6 Sf9 cells were seeded on the wells of a 6-well plate and 200 l of the eluted plaque-purified virus was used for infection, AII: 6×10^6 Sf9 cells were seeded on a 100-mm Petri dish and infected with 100 μ l of the AI virus, and AIII: 6×10^6 Sf9 cells were seeded on a 100-mm Petri dish and infected with 5–10 μ l of the AII virus. The titer of the AIII virus should be about 10^8 PFU (plaque forming units/ml).

*3.1.3. Expression of
Recombinant Human Type
III Procollagen in Insect
Cells (10)*

1. 6×10^6 H5 cells (*see Note 6*) were seeded on a 100-mm Petri dish and infected with the rhproCIII and 4PH viruses, the former was used in a five- to tenfold excess over the latter. Plates were incubated at 27°C and L-ascorbic acid phosphate (80 μ g/ml) was added to the culture medium daily.
2. Cells were harvested (*see Note 7*) 72 h after infection, washed with PBS and homogenized in the homogenization buffer (500 μ l/ 6×10^6 cells). The cell homogenates were centrifuged at $10,000 \times g$ for 20 min and the soluble fractions were collected. Samples were analyzed with SDS-PAGE under reducing conditions, followed by staining with Coomassie Brilliant Blue or Western blotting using polyclonal antibodies to the α and β subunits of human prolyl 4-hydroxylase (21), the N-propeptide of human type III procollagen (Farnos Diagnostica) or a monoclonal antibody 95D1A recognizing the collagenous region of various collagen chains (22). The amount of recombinant human type III procollagen was studied by means of a PIINP radioimmunoassay for the trimeric N-propeptide of human type III procollagen, and the amount of prolyl 4-hydroxylase activity was assayed

by a method based on the hydroxylation-coupled decarboxylation of 2-oxo[1- ^{14} C] glutarate (23).

3. The triple-helical conformation of the recombinant human type III procollagen was studied by pepsin digestion (24). The pH of the samples was lowered to 2.5, pepsin (1.5 mg/ml in 10 mM acetic acid) was added to a final concentration of 0.2 mg/ml, and the samples were digested for 1–4 h at 22°C. Pepsin was inactivated by adjusting the pH back to 7.4 and the samples were analyzed by SDS–PAGE (Fig. 1). The thermal stability of the pepsin digested recombinant human type III collagen was studied by trypsin–chymotrypsin digestion (25). Samples were preheated at the selected temperatures for 5 min and digested for 2 min with a mixture of 100 μ g/ml trypsin and 250 μ g/ml chymotrypsin. The digestion was terminated by adding trypsin inhibitor to a final concentration of 0.5 mg/ml and the samples were analyzed by SDS–PAGE.
4. Expression of recombinant human type III procollagen in H5 cells can be scaled up by using suspension cultures in shaker flasks or bioreactors. The largest amount of recombinant type III procollagen we have obtained from H5 cells cultured in shaker flasks has been about 60 mg/l (*see Note 8*). The recombinant type III collagen produced in H5 cells was found to be very similar in its 4-hydroxyproline content and T_m to type III collagen extracted from various tissues, whereas its hydroxylysine content was found to be about 60% of that of the nonrecombinant type III collagen (10).

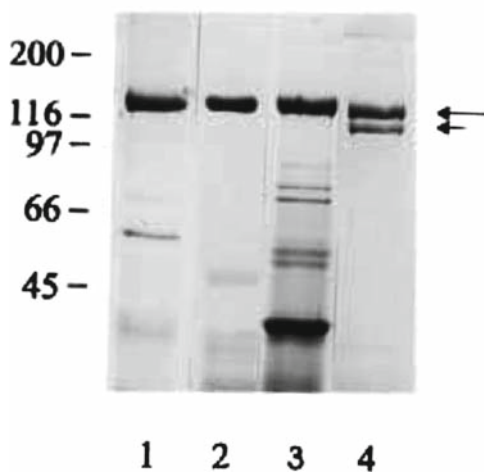


Fig. 1. SDS–PAGE analysis of pepsin digested recombinant human collagens expressed in H5 insect cells. Long arrow indicates the α 1 chains of type III (lane 1) and type II collagens (lane 2), the type I collagen homotrimer (lane 3), and the type I collagen heterotrimer (lane 4). Short arrow indicates the α 2 chain of type I collagen heterotrimer.

3.2. Expression of Recombinant Human Type III Procollagen in *Pichia pastoris*

3.2.1. Generation of Recombinant *Pichia* Expression Vectors (8)

1. A modified *Pichia* expression vector pARG815 was generated by replacing the *HIS4* selection marker in pAO815 with the *S. cerevisiae* *ARG4* selection marker. pYM25 containing the *ARG4* gene was digested with *HpaI*, and the *ARG4* *HpaI* fragment was ligated into *EcoRV*-digested pAO815.
2. The 5' end of the human prolyl 4-hydroxylase α subunit cDNA, extending from the translation initiation codon to an internal *HindIII* site, with *HindIII* and *SmaI* sites flanking the initiation codon, and the 3' end, extending from an internal *PstI* site to the translation stop codon, with *SmaI* and *BamHI* sites following the stop codon, were synthesized by PCR. These PCR fragments were used to replace the 5' and 3' ends of the original α subunit cDNA to generate a cDNA without any 5' and 3' untranslated regions. The *SmaI*–*SmaI* α subunit insert was ligated into the *EcoRI* site of pARG815, generating pARG815 α .
3. The signal sequence of the human prolyl 4-hydroxylase β subunit was replaced with the *S. cerevisiae* α mating factor (α MF) prepro sequence (see **Note 9**). Human prolyl 4-hydroxylase β subunit cDNA, extending from the codon for the first amino acid after the signal peptide cleavage site to the stop codon and flanked by *EcoRI* sites, was synthesized by PCR and ligated into the *EcoRI* site following the (α MF) prepro sequence of pPIC9, generating pPIC9 β .
4. The 3' end of the pro α 1(III) cDNA used in the generation of the recombinant baculovirus pVLRhproCIII (see **step 2** in **Subheading 3.1.1**) was replaced by a PCR fragment extending from an internal *EcoRI* site to the translation stop codon, with a *XbaI* site following the stop codon. The *BglII*–*XbaI* pro α 1(III) cDNA was ligated into the *EcoRI*–*XbaI* site of pPICZ B, generating pPICZ Bpro α 1(III).

3.2.2. Generation of the Recombinant *Pichia* Strain and Expression of the Recombinant Human Type III Procollagen (8, 19)

1. To obtain a recombinant *P. pastoris* strain expressing human prolyl 4-hydroxylase, pARG815 α and pPIC9 β were linearized with *DraIII* and *StuI*, respectively, and cotransformed into the *his4*, *arg4* *P. pastoris* host strain by the electroporation method (19). Five milliliters of the *his4*, *arg4* *P. pastoris* host strain was cultured in YPD in a 50-ml conical tube at 30°C overnight. Five hundred milliliters of YPD was inoculated with 0.1–0.5 ml of the overnight culture and grown to an OD₆₀₀ of 1.3–1.5 (see **Note 10**). The cells were centrifuged at 4°C at 1,500 $\times g$ for 5 min and washed twice, with 500 ml and 250 ml of ice-cold, sterile water. They were then resuspended in 20 ml of ice-cold 1 M sorbitol, centrifuged, and resuspended in ice-cold 1 M sorbitol to obtain a final volume of 1.5 ml. Three microliters (approximately 0.6 μ g) of the linearized pARG815 α and pPIC9 β were mixed with 40 μ l of the *P. pastoris* cells, transferred to an ice-cold electroporation

cuvette and incubated on ice for 5 min. The cells were pulsed using the parameters 1,500 kV, 25 μ F, and 400 Ω (*see Note 11*), and 1 ml of ice-cold 1 M sorbitol was added immediately afterward. Aliquots of 50–200 μ l of the cells were spread on MD plates and incubated at 30°C until colonies appeared. Selection for His⁺, Arg⁺ colonies was repeated twice by streaking single colonies on MD plates.

2. The recombinant His⁺, Arg⁺ colonies obtained above were cultured at 30°C in 25 ml of BMGY in 250-ml shaker flasks to an OD₆₀₀ of 5–10. The cells were centrifuged at 1,500 $\times g$ for 5 min and resuspended to an OD₆₀₀ of 1–3 in BMM to induce expression. Methanol was added every 24 h to a final concentration of 0.5%. Cells were harvested after a 60-h methanol induction, washed once and resuspended to an OD₆₀₀ of 250–350 in cold 5% glycerol in 50 mM sodium phosphate buffer, pH 7.4. An equal volume of glass beads was added and the samples were vortexed eight times for 30 s at 4°C with 30 s intervals. They were then centrifuged at 15,000 $\times g$ for 30 min at 4°C, the supernatants were collected and screened for expression of the prolyl 4-hydroxylase α and β **step 2** of **Subheading 3.1.3**. A recombinant strain expressing prolyl 4-hydroxylase was selected and termed $\alpha/\beta\alpha$ -MF.
3. A recombinant strain coexpressing human prolyl 4-hydroxylase and pro α 1(III) chains was generated by transforming *Pme*I-linearized pPICZ Bpro α 1(III) into the $\alpha/\beta\alpha$ -MF strain by electroporation as in **step 1**. The transformants were selected in YPD (+100 μ g/ml zeocin) (*see Note 12*). The cells were cultured, induced and harvested as in **step 2**, and expression of human prolyl 4-hydroxylase and type III procollagen was assayed in the soluble fraction of cell lysates as in **step 2** of **Subheading 3.1.3** (*see Note 13*). A recombinant strain producing human type III procollagen was selected based on these assays and termed $\alpha/\beta\alpha$ -MF/pro α 1(III).
4. For the production of recombinant human type III procollagen, the $\alpha/\beta\alpha$ -MF/pro α 1(III) *Pichia* strain was cultured in shaker flasks and induced with methanol. Cells were harvested 60 h after methanol induction and broken with glass beads as in **step 2**. The purified recombinant type III collagen produced in shaker flasks was essentially identical in its amino acid composition to nonrecombinant human type III collagen, except that the degree of 4-hydroxylation of the proline residues was 44.2% while the corresponding value for nonrecombinant type III collagen is 51.6% (8). However, essentially fully hydroxylated recombinant human procollagens can be produced in *P. pastoris* in bioreactors under optimal oxygenation conditions (13). The *Pichia*-derived recombinant human type III collagen contains no hydroxylysine, whereas nonrecombinant type III collagen has five hydroxylysine residues per

1,000 amino acids (8). Expression levels of stable recombinant human collagens have ranged up to 1.5 g/l in *P. pastoris* in bioreactor cultures (3, 4).

4. Notes

1. In the p2Bac and pVL1392 vectors the ATG translation initiation codons of polyhedrin and p10 have been altered to ATT, which means that the inserts must provide their own ATG initiation codons. The 5' cloning site in the inserts should be as close to the translation initiation codon as possible (less than 100 bp) (20). Since translation starts from the first ATG codon downstream of the polyhedrin and p10 promoters, no additional ATG codons should exist upstream of the initiation codon of the gene of interest. For example, the polyhedrin multicloning site in the p2Bac vector has a *NcoI* recognition sequence (CCATGG), and therefore none of the downstream cloning sites can be used. The length or sequence of the 3' untranslated region of an insert usually has no effect on the expression levels. The p2Bac and pVL1392 vectors provide the polyhedrin or SV40 polyadenylation signals, and therefore the genes of interest do not have to include any polyadenylation signals.
2. BaculoGold DNA is a modified baculovirus DNA which contains a lethal deletion (20), and viable virus particles are obtained only by cotransfection with a complementing baculovirus expression vector. When BaculoGold DNA is used, the expression vectors must therefore include at least 1.7 kbp of baculovirus DNA downstream of the polyhedrin stop codon to counteract the lethal deletion. The recombination frequency with BaculoGold DNA is at least 99%, which is a major improvement over the 0.1% recombination frequency obtained with the wild-type baculovirus DNA.
3. In spite of the high recombination frequency with the BaculoGold DNA and the fact that wild-type baculovirus DNA expressing polyhedrin cannot be formed during recombination, it is still advisable to plaque-purify the recombinant virus stocks, as aberrant crossing over during transfection can lead to total or partial deletions of the recombinant gene of interest. The resultant virus stock after plaque-purification is derived from a single virus clone.
4. If plaques are not clearly visible, the plaque assay plates can be stained with Neutral Red or MTT (thiazolyl blue), for example (26).
5. The MOI (multiplicity of infection = plaque-forming units/cell number) should be below 1 during all amplifications, as

values greater than 1 can lead to deletions in the recombinant virus particles (20). The PFU/ml of a virus stock can be obtained from a plaque assay by the formula: PFU/ml = 1/virus dilution (e.g., 10^{-5}) $\times$ number of plaques $\times$ 1/ml virus dilution added to the plate.

6. H5 cells consistently give three- to tenfold higher expression levels than Sf9 cells for recombinant human type III procollagen (10).
7. Most of the recombinant fibril-forming human type I, II, or III procollagens produced (70–90%) is retained within the insect cells (10–12).
8. The highest expression levels obtained for type I and II collagens in suspension culture have been 20–40 and 50 mg/l, respectively (11, 12).
9. The signal sequence of the prolyl 4-hydroxylase β subunit was found to play a major role in enzyme assembly in *Pichia* (8). The authentic human signal sequence was ineffective for transport of the β subunit into the lumen of the endoplasmic reticulum, as only trace amounts of an active $\alpha_2(\beta)_2$ enzyme tetramer were produced. The highest tetramer assembly level was obtained with the *S. cerevisiae* α mating factor pre-pro sequence, while that obtained with the *P. pastoris* acid phosphatase 1 signal sequence was about 40% of this value.
10. The doubling time of the *his4, arg4 P. pastoris* strain is approximately 2 h in YPD.
11. *P. pastoris* cells can be pulsed using the parameters suggested for *S. cerevisiae* by the manufacturer of the electroporation device.
12. Zeocin is active only at a low salt concentration (<90 mM) and at pH 7.5. Therefore, screening for His⁺, Arg⁺ phenotype and zeocin resistance must take place in parallel plates, as the pH of MD plates is not suitable for zeocin selection.
13. As in the case of insect cells (see Note 7), the vast majority of the recombinant human type III procollagen in the *Pichia* expression system, too, is retained within the cells and can thus be obtained from the soluble fraction of the cell lysates (8).

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