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ORIGINAL ARTICLE

Production of human type II collagen using an efficient baculovirus-silkworm multigene expression system

Qi Qi[1] · Lunguang Yao[1,2] · Zhisheng Liang[3] · Donghua Yan[1] · Zhuo Li[4] · Yadong Huang[3] · Jingchen Sun[1]

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Abstract Human type II collagen is a macromolecular protein found throughout the human body. The baculovirus expression vector system is one of the most ideal systems for the routine production and display of recombinant eukaryotic proteins in insect, larvae, and mammalian cells. We use this system to express a full-length gene, human type II collagen cDNA (4257 bp), in cultured *Spodoptera frugiperda* 9 cells (*Sf*9), *Bombyx mori* cells, and silkworm larvae. In this study, the expression of human type II collagen gene in both insect cells and silkworm larvae was purified by nickel column chromatography, leading to 300-kDa bands in SDS-PAGE and western blotting indicative of collagen α-chains organized in a triple-helical structure. About 1 mg/larva human type II collagen is purified from silkworm skin, which shows a putative large scale of collagen production way. An activity assay of recombinant human type II collagen expressed by silkworm larvae demonstrated that the recombinant protein has considerable bioactive properties. Scanning electron microscopy of purified proteins clearly reveals randomly distributed and pitted structures. We conclude that the baculovirus-silkworm multigene expression system can be used as an efficient platform for express active human type II collagen and other complicated eukaryotic proteins.

Keywords Human type II collagen · BEVS · *Bombyx mori* · *Sf*9 · Triple-helix

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Q. Qi, L. Yao, and Z. Liang contributed equally to this work.

✉ Jingchen Sun
cyfz@scau.edu.cn

[1] Subtropical Sericulture and Mulberry Resources Protection and Safety Engineering Research Center, Guangdong Provincial Key Laboratory of Agro-animal Genomics and Molecular Breeding, College of Animal Science, South China Agricultural University, Guangzhou 510642, Guangdong, China

[2] Henan Key Laboratory of Ecological Security for Water Source Region of Mid-line Project of South-to-North Diversion, Nan Yang Normal University, Nanyang 473000, Henan, China

[3] Institute of Biomedicine, Jinan University, Guangzhou 510632, Guangdong, China

[4] Medical Technology College of Nanyang Medical University, Nanyang 473041, Henan, China

Introduction

Collagens are the most abundant proteins in mammals including humans which are about 30 % of total protein mass. Collagen plays important roles as not only a scaffold for the cell attachment and migration, but also a regulator for the growth and differentiation of cells occurring in embryogenesis, regeneration, and wound healing (Yoshizato et al. 1984; Yoshizato and Makino 1988; Iino et al. 1996; Asahina et al. 1999). Collagens are structural proteins present in extracellular matrices of all tissues (McAlinden et al. 2014), and type II collagen is the most abundant collagen in articular cartilage, together with other tissue-specific collagens and proteoglycans, provides the tissue with its shock-absorbing properties and its resiliency to stress, and comprises approximately 50 % of the extracellular matrix of the tissue (Eyre 1991; Bruckner and van der Rest 1994). Type II collagen is the main component of hyaline cartilage and considered to be one of the most important of rheumatoid arthritis (RA) autoantigens (Sapadin and Fleischmajer 2006; Shahab et al. 2012). Type

II collagen is most likely the RA target antigen, because it can stimulate T-cell-mediated immune responses in the incidence of RA (Min et al. 2004). In addition, type II collagen is also an important component of the cornea. Collagen fibrils arranged regularly in the cornea allow light to pass through, thereby being responsible for the transparency of the cornea (Wollensak and Iomdina 2009).

Collagen production is mainly by means of acidolysis and alkaline hydrolysis or enzymatic extraction from animal connective tissue at present, such as pig skin, cattle hide, donkey skin, fish, and so on (Dormont 1999). Its biological activity is somewhat lost in the process of extraction. Furthermore, the extracted animal-derived product can often produce allograft rejection, when it was applied to the human body. In addition, collagen extracted from animal tissue is water insoluble protein. It is impossible to dissolve without changing the molecular weight or structure of the protein, which has adverse effects for workability and plasticity, thus limiting the applicability of the production of collagen. Although the method of chemical synthesis of collagen can solve the above problem, collagen produced in such a manner usually lacks the necessary hallmarks of biological activity structures (i.e., triple-helix configurations), and synthesis technology is relatively complex and high cost. The FDA and European Union have placed restrictions on the medical applications of animal-sourced collagen, so looking for a safe, biologically effective method sourcing of collagen has garnered increasing attention in the field of tissue engineering, medicine, and cosmetics.

With the development of genetic engineering technology, many kinds of host cells were used to produce heterologous recombinants. Type I, II, and III collagens were successfully expressed in *Pichia pastoris* (Vuorela et al. 1997). The human type II collagen alpha-chain and α/β-prolyl 4-hydroxylases coding sequences were co-expressed in insect cells to produce a stable triple-helix human type II collagen with a yield of up to 50 mg/L using two baculovirus co-infection *Sf*9 cells (Nokelainen et al. 1998). The recombinant collagen expressed with heterologous expression system not only had good biocompatible, biodegradable, and cell adhesion characteristics, but also could promote epithelial cell formation and stop bleeding. In addition, the workability of collagen was superior to collagen of animal origin (Lynn et al. 2004; Kotch and Raines 2006). Because the human type II collagen alpha single chain cDNA has 4257 bp, it is difficult to express using a prokaryotic expression system. In comparison, the yeast expression system possesses slightly higher post-translational processing and glycosylation modification capability, yet it is not as capable as an insect cell expression system, which contains unique characteristics well suited to our purposes (Altmann et al. 1999). Compared with the yeast expression system, it has lower background interference and an even stronger ability to carry out post-translational processing and modification (Ikonomou et al. 2003). Compared with the mammalian expression system, insect cell expression systems have many advantages, such as lower requirements, in environmental requirements and more easily cultured. In addition, the mammalian proteins expressed by insect cell expression can be correctly folded and contain no endogenous toxin (Kidd and Emery 1993).

Accordingly, we chose the baculovirus expression system to express the complete human type II collagen gene, including alpha peptide and triple-helix structures. Much research and development has been invested in creating a streamlined recombinant BmNPV, which has yielded systems, such as the BmNPV-based Bac-to-Bac system, the mating-assisted genetically integrated cloning (MAGIC) method, and a zero-background Tn7-mediated transposition-based method in *E. coli* (Yao et al. 2010). In our previous work, we have improved some aspects of the construction of recombinant BmNPV and we combined those different methods to establish another efficient MultiBac system named BmMNPV-silkworm multigene expression system (Yao et al. 2012). In this paper, we used silkworm larvae as a host production of biologically active human type II collagen.

Materials and methods

Bacterial strains, plasmids, viral bacmid, reagents, and larvae

Escherichia coli TOP10 was used for the propagation of normal plasmids, such as pFBDM. *E. coli* sw106 was provided by Prof. Copeland. Plasmid EX-Z5512-B31 containing human type II collagen cDNA was provided by Prof. Wang Jufang. Spectinomycin (spe)-resistance plasmid pGB2inv-hly (containing both hly and inv genes) was provided by Prof. Courvalin. The modified BmBacmid with mini-attTn7 in lacZ α gene was constructed at our previous study (Yao et al. 2012), Pfu*Taq* was purchased from TaKaRa, restriction enzymes were purchased from NEB (New England BioLabs), and T_4 DNA ligase was purchased from Fermentas, while DL-a-e Diaminopimelic acid (DAP) was bought from Sigma (cat. D1377, USA). Grace's Insect Cell Culture Medium and Fetal Bovine Serum were purchased from Gibco. Silkworm variety is 9·Fu × 7·Xiang.

Construction of the baculovirus transfer vectors

Human type II collagen has been assumed to be two types, transcript variant 1 and transcript variant 2, which have been published on NCBI (NCBI Reference Sequences: NM_001844.4 and NM_033150.2, found in cartilage and the vitreous humor of the eye). This variant 2 lacks an

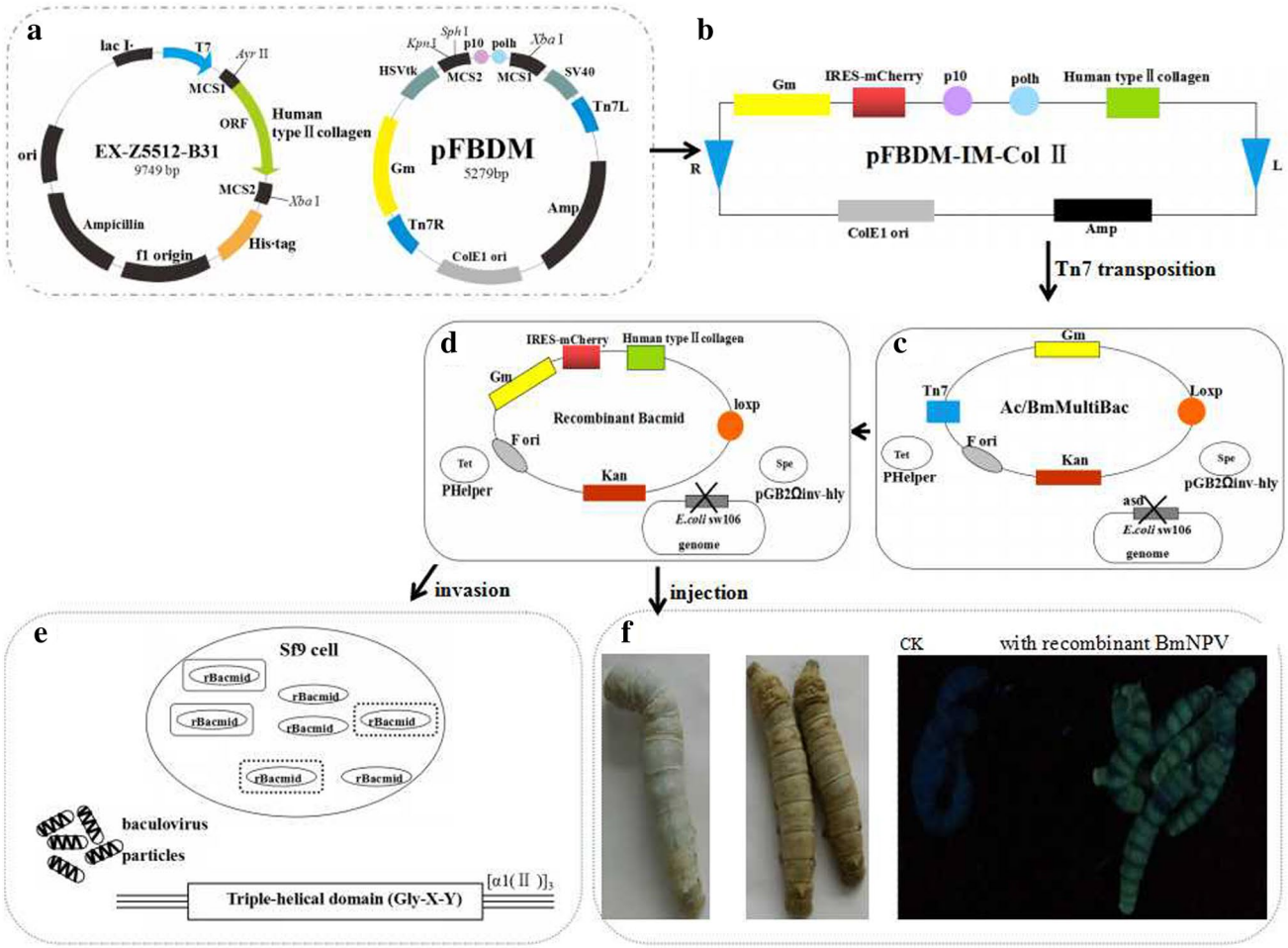

Fig. 1 Construction of recombinant baculovirus with human type II collagen gene using multigene expression system. **a** Construction information of plasmid EX-Z5512-B31 and pFBDM. The plasmid pFBDM contains two expression cassettes in a head-to-head arrangement with multiple cloning sites MCS1 and MCS2 flanked by polh or p10 promoters and SV40 or HSVtk polyA signal sequences, respectively. The sequences used for Tn7 transposition (Tn7L and Tn7R) encompass the expression cassettes and antibiotic resistance genes (Amp, Gm) for screening. The plasmid EX-Z5512-B31 contains the human type II collagen cDNA. **b** Transfer vector was named as pFBDM-IM-colII, in which the polyhedrin promoter (polh) directs the synthesis of human type II collagen, while p10 promoter controls the synthesis of IM. **c**, **d** Type II collagen gene and IM gene can be introduced into Bacmid through mini-Tn7 transposon. **e** Invasive DAP auxotrophic *E. coli*, which contains recombinant AcMultiBac, was directly added into *Sf*9 cells for the generation of recombinant baculovirus in insect cells. **f** Invasive DAP auxotrophic *E. coli*, which contains recombinant BmMultiBac, was injected into silkworm larval hemocoel for production of recombinant baculovirus. Most of the injected larvae (90 %) showed fluorescence in sunlight 4 days post injection when observed with gel imaging system

alternate in-frame exon in the 5′ coding region, compared with variant 1, resulting in a shorter protein. The resulting protein (isoform 2) lacks the von Willebrand factor type C (VWC) domain, compared with isoform 1. The transfer vector was constructed using these coding sequences in EX-Z5512-B31 (Fig. 1) which contain variant 2 of human type II collagen fusion with 6 × His tag sequences. The plasmid EX-Z5512-B31 was digested with *Avr* II/*Xba* I and ligated into pFBDM (multiple cloning sites MCS1) digested with *Xba* I, and IRES-mCherry was ligated into pFBDM (multiple cloning sites MCS2) digested with *Sph* I/*Kpn* I, respectively. Then, the transfer vector was named as pFBDM-IM-colII, in which the mCherry report gene is translated by an internal ribosome entry site (Fig. 1).

Introduction of multiple genes into Bacmid

The recombinant plasmid pFBDM-IM-colII was introduced into *asd*-deletion type of *E. coli* sw106-inv components contained AcMultiBac or BmMultiBac by Tn7 transposition as described in a previous study (Sun et al. 2009, 2010). The clone grown on Gm/Kan/Tet/Spe/DAP was screened by white-blue plaque selection and PCR screening. The bacteria of sw106-Ac-pFBDM-IM-colII

and sw106-Bm-pFBDM-IM-colII were grown in 5-mL LB broth supplement with 5-μg/mL DAP, 5-μg/mL spectinomycin, 50-μg/mL kanamycin, 5-μg/mL tetracyclines, and 7-μg/mL gentamicin at 37 °C, respectively.

Production of the recombinant viruses

Sw106 cells with recombinant Bacmid were cultured to OD_{600} (attenuance at 600 nm) 1.0 and collected by centrifugation. The supernatant was removed and the bacterial pellet was resuspended in distilled ultrapure water for three times. The pellet was resuspended in 1 mL of Grace's insect medium. The bacterial suspension was adjusted to different densities (10^5–10^8 cells/mL) (Yao et al. 2010) with Grace's insect medium. Insect cells were incubated overnight in a 24-well plate (60–70 % confluent single layer). After removing the medium, 500 μL of sw106-Ac-pFBDM-IM-colII and sw106-Bm-pFBDM-IM-colII bacterial suspension at different densities was added to each well to infect *Sf*9 insect cells and ovary cells of *Bombyx mori* (BmN), respectively. Five hundred microlitre of fresh complete Grace's insect medium (with 10 % FBS and 0.075 % Penicillin Streptomycin) was incubated for 4 h in each well, and insect cells were then incubated for 3–5 days with fresh complete Grace's insect medium. The recombinant baculovirus was termed AcMNPV-pFBDM-IM-colII in *Sf*9 insect cells and BmNPV-pFBDM-IM-colII in BmN cells. The mCherry fluorescence was assayed by fluorescence microscopy after 3–5 days, which was interpreted as being indicative of successful target gene expression.

Expression of recombinant human type II collagen in insect cells

*Sf*9 insect cells were cultured in Grace's insect medium supplemented with 10 % FBS and 0.075 % Penicillin Streptomycin monolayers. The *Sf*9 cells were allowed to grow at 28 °C for 3 days and the cultural cell supernatant without insect cells was collected as the primary viral stock with a high titer (10^8 pfu/mL). The *Sf*9 cells were harvested 72 h after infection, assayed by fluorescence, washed and homogenized with a solution of 0.2 M NaH_2PO_4 and 0.2 M Na_2HPO_4 (PB buffer), pH 6.0, preserved at −20 °C until completely frozen, melted slowly, and centrifuged at 12,000*g*, 4 °C for 10 min. The *Sf*9 cells and supernatant were separated at 4 °C after assaying for fluorescence. The same process was applied to BmN cells.

SDS-PAGE and western blotting of collagen in insect cells

Aliquots of the supernatants of cell homogenates and cell culture supernatant were analyzed by 8 % SDS-PAGE under reducing conditions followed by Coomassie staining or western blotting with anti-His antibody recognizing a His tag attached to the type II collagen coding sequences. Goat Anti-Mouse IgG-HRP antibody was used as a second antibody.

Purification of collagen in *Sf*9 insect cells

The virus AcMNPV-pFBDM-IM-colII was added into a 75-cm^2 cell culture flask (400 μL/bottle) with monolayer *Sf*9 cells spread to more than 80 % of the bottom. *Sf*9 cells were harvested after 72-h infection, assayed for fluorescence, washed, and homogenized with a solution of 0.2 M PB buffer (6 mL/bottle), pH 6.0, added 17-μg/mL phenylmethanesulfonyl fluoride (PMSF) to inhibit serine protease and cysteine proteinase, broke up at 9 W for 1 min by Selecta Sonopuls, and centrifuged at 12,000*g*, 4 °C for 10 min. Unless otherwise mentioned, all the following steps were performed at 4 °C. The sample of cells was added with PMSF, diluted three times by 0.2-M PB buffer (pH 6.0), and filtered by 0.45-μm pore size nucleopore filter. The diluted sample was then filtered with an ultrafiltration device (100,000 MWCO). The sample was concentrated about eight times. The sample (>100 kD) was added to PMSF and then ran on Ni–NTA affinity columns equilibrated with 20-mM PB buffer, pH 6.0 (buffer A), and eluted with 20-mM PB, 200-mM NaCl, 0.5-M imidazole buffer, and pH 6.0 (buffer B). Miscellaneous (non-target) protein was eluted with 20-mM PB, 200-mM NaCl, and 50-mM imidazole buffer (pH 6.0), and target protein was eluted with 20-mM PB, 200-mM NaCl, and 150-mM imidazole buffer (pH 6.0) by NGC (Bio-Rad). All collected aliquots were then analyzed by 8 % SDS-PAGE followed by Coomassie staining or western blotting with anti-His antibody recognizing the His tag of type II collagen.

Production of collagen in silkworm larvae

The newly molted fifth-instar silkworm larvae were used for infection and expression. The virus of BmNPV-pFBDM-IM-colII was prepared from BmN cell culture after the virus reached a sufficiently high titer. Two microlitre of recombinant virus was injected into the dorsal haemocoel for each larva using a syringe with a 100 μL micro-injector. Half an hour later, the larvae were fed with fresh mulberry leaves and reared under constant environmental conditions of 28 °C, and humidity between 60 and 70 %. The silkworm larvae were allowed to grow for 4 days, after which the haemolymph and skin were collected for analysis by 8 % SDS-PAGE and western blotting.

Purification of collagen in silkworm larvae

A total of 50 larvae were injected with the recombinant virus and monitored every day until they showed the symptoms of

NPV infection. Larval scolex and telson were cut, the midgut and silk gland were peeled, and the skin was kept as primary sample for the purification of recombinant type II collagen. The skin was stirred and crushed by a high-speed homogenate machine for 12 times with 10-s interval and 20-s gap while in a trypsin inhibitor and 0.2-M PB buffer (pH 6.0) solution (all steps were performed on ice). The sample was then processed by pressure homogenization at 300 MPa. The sample was collected and centrifuged at 18,000*g* for 20 min. Chromatography was then carried out on the sample supernatant using a nickel column equilibrated with 20-mM PB and 200-mM NaCl buffer (pH 6.0). As before, miscellaneous non-target protein was eluted with 20-mM PB, 200-mM NaCl, and 50-mM imidazole buffer (pH 6.0), while target protein was eluted with 20-mM PB, 200-mM NaCl, and 150-mM imidazole. Target protein was then run on G25 desalting column with 20-mM PB and 200-mM NaCl buffer (pH 6.0), and kept in the same buffer. The 50-mM and 150-mM imidazole elution buffer was collected and analyzed by 8 % SDS-PAGE followed by Coomassie or silver staining and western blotting with anti-His antibody. The desalted target protein sample was then lyophilized at −80 °C under vacuum conditions.

MTT detection of collagen expressed by silkworm larvae

The lyophilized type II collagen sample was dissolved with ultrapure water, filtered by 0.22 μm microporous membrane, added in a 96-well plate (40 μL/well), and dried in fume hood. Bovine fibronectin was used as positive control and saline as negative control. Balb/c 3T3 cells were washed by PBS buffer, incubated with 0.02 % EDTA and 0.25 % trypsin, and collected by centrifugation. The Balb/c 3T3 cells (1.6×10^5 cells/mL) were resuspended and added into well-contained blow-dried type II collagen samples overnight in 1640 medium with 10 % FBS, 37 °C for 4–5 h. Carbon dioxide concentration was controlled at 5 %. Redundant cells were then washed by PBS buffer. The remaining cells were then gently washed using 100 μL PBS buffer. After removing PBS buffer, 100 μL 1640 medium was added to each well with 10 % FBS for 4 h. After 4 h of incubation, 10 μL of MTT was added to the Balb/c 3T3 cells in each well and incubated for another 2–4 h. The medium was then removed, and 100 μL of terminal solution DMSO was added to each well, after which the wells were kept at room temperature for 30 min. ELISA was used to detect each well's absorption at 570- and 630-nm wavelengths.

Observation with scanning electron microscope

The lyophilized type II collagen sample was then critical-point dried, mounted, and stained. Observation and subsequent micrographs were taken on an XL 30 scanning electron microscope (SEM).

Results

Construction of the AcNPV-Sf9 and BmNPV-BmN multigene expression system

The AcNPV-*Sf*9 and BmNPV-BmN multigene expression systems consist of the modified recipient strain *E. coli* sw106MultiAcBac and *E. coli* sw106MultiBmBac, and the transfer vector pFBDM. The type II collagen gene and IM gene can be introduced into Bacmid through mini-Tn7 transposons. The recombinant plasmid pFBDM-IM-colII was introduced into asd-deletion type of *E. coli* sw106-inv component cell containing either AcMultiBac or BmMultiBac by Tn7 transposition. The colonies grown on Gm/Kan/Tet/Spe/DAP were screened by white-blue plaque selection and PCR detection. The recombinant bacmid was then prepared and transfected into cultured insect cells, *Sf*9 or BmN. When *E. coli* sw106 with invasin protein incubated with insect cells, sw106 can infect the insect cells. Sw106 can not replicate/reproduce under the absence of DAP and die in the insect cells. Four-day post-infection, insect cells infected with recombinant virus are observed under 543-nm wavelength light (mCherry). These results indicated that the AcMNPV-pFBDM-IM-colII and BmMNPV-pFBDM-IM-colII recombinants were constructed successfully (Fig. 1).

Expression of recombinant human type II collagen in insect cells and silkworm larvae

As shown in Fig. 2, both the cultured cells and silkworm larvae turned red, which indicated that the recombinant viruses were produced and the mCherry was expressed as expected (Fig. 2). To confirm the expression of human type II collagen, the culture medium and insect cells (*Sf*9 and BmN) were collected separately and kept at −20 °C until completely frozen, melted slowly, and centrifuged at 12,000*g* at 4 °C for 10 min. SDS-PAGE followed by Coomassie Brilliant Blue staining showed the presence of a major band corresponding to the triple-helical structure of type II collagen α-chains in *Sf*9 cells and BmN cells at 300 kDa (Fig. 3). Accordingly, the western blotting analysis proved that the target proteins were detected using anti-His tag antibody. This indicated that recombinant human type II collagen was successfully expressed in *Sf*9 cells and BmN cells.

Four days later, the haemolymph was collected for sampling and analysis by 8 % SDS-PAGE. There was a

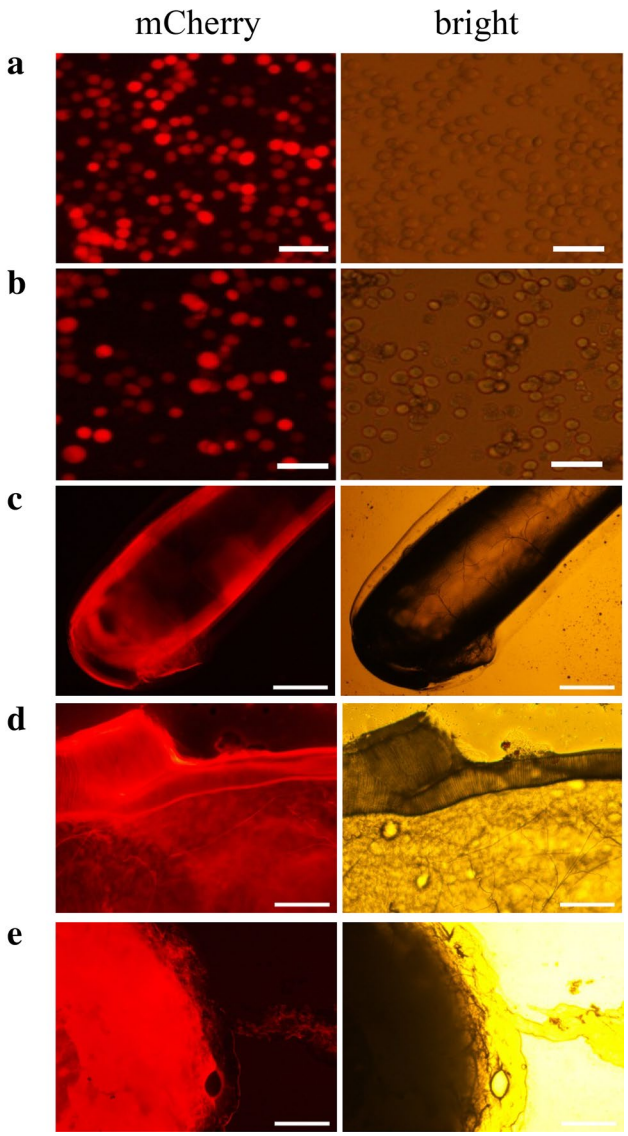

Fig. 2 Observation of insect cells and different tissue of silkworm larvae infected by recombinant virus under Nikon TS100. **a** *Sf*9 cells infected with recombinant virus AcMNPV-pFBDM-IM-colII after 72 h. **b** BmN cells infected with recombinant virus BmMNPV-pFBDM-IM-colII after 72 h. The images of cells were taken at the *bright and red* (590 nm). *Bar* 50 μm. **d**, **e** Silkworm epidermis. The tissues **c** sericterium; **d**, **e** epidermis from silkworm larvae infected with recombinant virus BmMNPV-pFBDM-IM-colII was observed using Nikon TS100. The tissue images were taken at the *bright* and *red* (590 nm). *Bar* 500 μm

major band of the haemolymph sample, corresponding to the triple-helical structure of type II collagen α-chains at approximately 300 kDa. As expected, the control of haemolymph extracted from silkworm larvae did not show the corresponding band (Fig. 3). In addition, the western blotting analysis results showed the expressed protein fusion with a His tag as expected when using anti-His tag antibody.

Purification of recombinant human type II collagen

*Sf*9 cells were collected and suspended with PB buffer (pH 6.0), treated as described earlier in "Materials and methods". Then, the recombinant protein with His tag was purified by Ni–NTA affinity columns. The elution buffer was analyzed using 8 % SDS-PAGE followed by Coomassie Brilliant Blue staining. The results demonstrated that elution buffer, including 150 mM imidazole, could elute the target protein. The band corresponding to the triple-helical structure of type II collagen α-chains was measured to be approximately 300 kDa (Fig. 4). The results showed that the triple-helical structure of type II collagen α-chains were easily broken, as seen from a major band corresponding to non-triple-helical type II collagen α-chains at approximately 130 kDa (Fig. 4).

The skin of 50 larvae infected with BmMNPV-pFBDM-IM-colII was collected and reserved in 10-mL PB buffer. The recombinant protein was purified by Ni–NTA affinity columns. SDS-PAGE and western blotting results showed the presence of major bands corresponding to both single and triple-helical α-chains. Type II collagen α-chains presented at approximately 130 kDa, while triple-helical α-chains presented at approximately 300 kDa (Fig. 4). The results indicated that our BmMultiBac expression system was able to express the human type II collagen in silkworms larvae.

Activity analysis of recombinant human type II collagen expressed by silkworm larvae

Different concentrations (0.6, 0.4, and 0.2 μmol/L) of purified human type II collagen sample were used in the experiments of cell adhesion by mixed with Balb/c 3T3 cells. 0.1-μM bovine fibronectin was used as a positive control, while saline was used as negative control. ELISA results showed that 0.6-μM recombinant protein reached OD = 0.211, which could prove that recombinant human type II collagen, expressed by silkworm larvae, has remarkable bioactivity (Fig. 5).

Electron microscope of triple-helical structure of recombinant human type II collagen

The scanning electron microscope (SEM) image showed that the purified recombinant human type II collagen has typical pitted structures (Fig. 6), which further supports

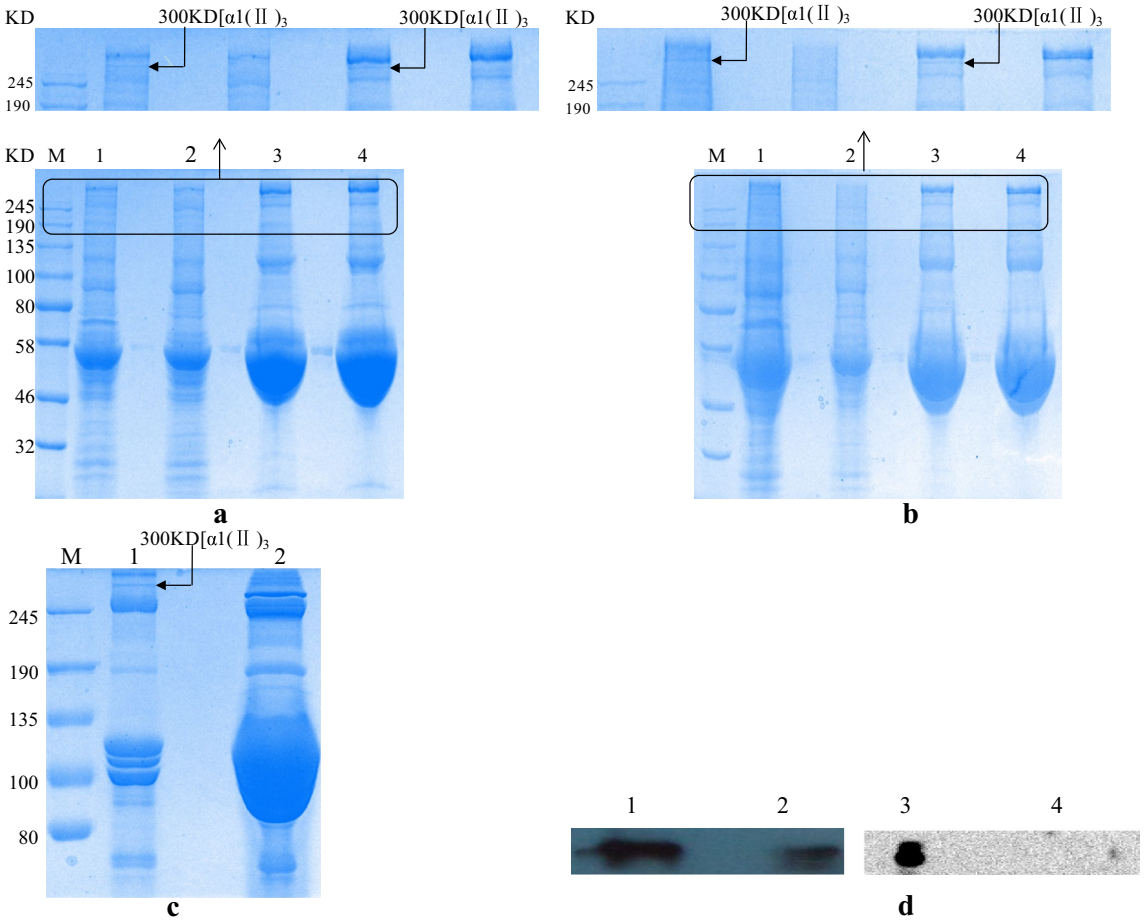

Fig. 3 SDS-PAGE and western blot analysis of human type II collagen expressed in insect cells and silkworm haemolymph. **a** Expression of recombinant human type II collagen in *Sf*9 cells on 8 % SDS-PAGE. *Lane M* color protein standard, broad range. *Lane 1 Sf*9 cells infected with the recombinant AcMNPV-pFBDM-IM-colII; *lane 2* non-infected *Sf*9 cells; *lane 3* complete Grace insect medium of *Sf*9 cells infected with the recombinant AcMNPV-pFBDM-IM-colII; *lane 4* complete Grace insect medium of non-infected *Sf*9 cells. The $[\alpha1(II)]_3$ was labelled. **b** Expression of recombinant human type II collagen in BmN cells on 8 % SDS-PAGE. *Lane M* color protein standard, broad range. *Lane 1* BmN cells infected with the recombinant BmMNPV-pFBDM-IM-colII; *lane 2* non-infected BmN cells; *lane 3* complete Grace insect medium of BmN cells infected with the recombinant BmMNPV-pFBDM-IM-colII; *lane 4* complete Grace insect medium of non-infected BmN cells. The $[\alpha1(II)]_3$ was labelled. **c** Expression of recombinant human type II collagen in silkworm larvae. *Lane M* color protein standard, broad range. *Lane 1* haemolymph infected with the recombinant BmMNPV-pFBDM-IM-colII; *lane 2* haemolymph non-infected with virus. The $[\alpha1(II)]_3$ was labelled. **d** Western blot analysis of the recombinant human type II collagen using anti-His tag monoclonal antibody. *Lane 1* sample from *Sf*9 cells infected with recombinant virus; *lane 2* sample from BmN cells infected with recombinant virus; *lane 3* haemolymph sample from larvae infected with recombinant virus, *lane 4* haemolymph sample from non-infected silkworm larvae (color figure online)

that the protein expressed in silkworm is a kind of typical collagen protein.

Discussions

The BmNPV-silkworm larvae expression system is much more attractive than the AcMNPV-*Sf*9 system mainly due to the lower cost of breeding silkworms and the higher levels of expression (Yao et al. 2010). We used the new baculovirus expression system to express the human type II collagen gene. The data reported here indicate that it is possible to obtain cartilage-specific human type II collagen with a stable triple helix in insect cells and a much higher expression level can be obtained using silkworm larvae as the host. This expression level is likely to be sufficient for various experimental and clinical applications of the recombinant protein. In this study, about 50 mg of biologically active human type II collagen was purified from 50 infected larvae skin, which indicated that the expression level of human type II collagen is quite high in silkworm. The approach established here is most economical and efficient way of producing human type II collagen.

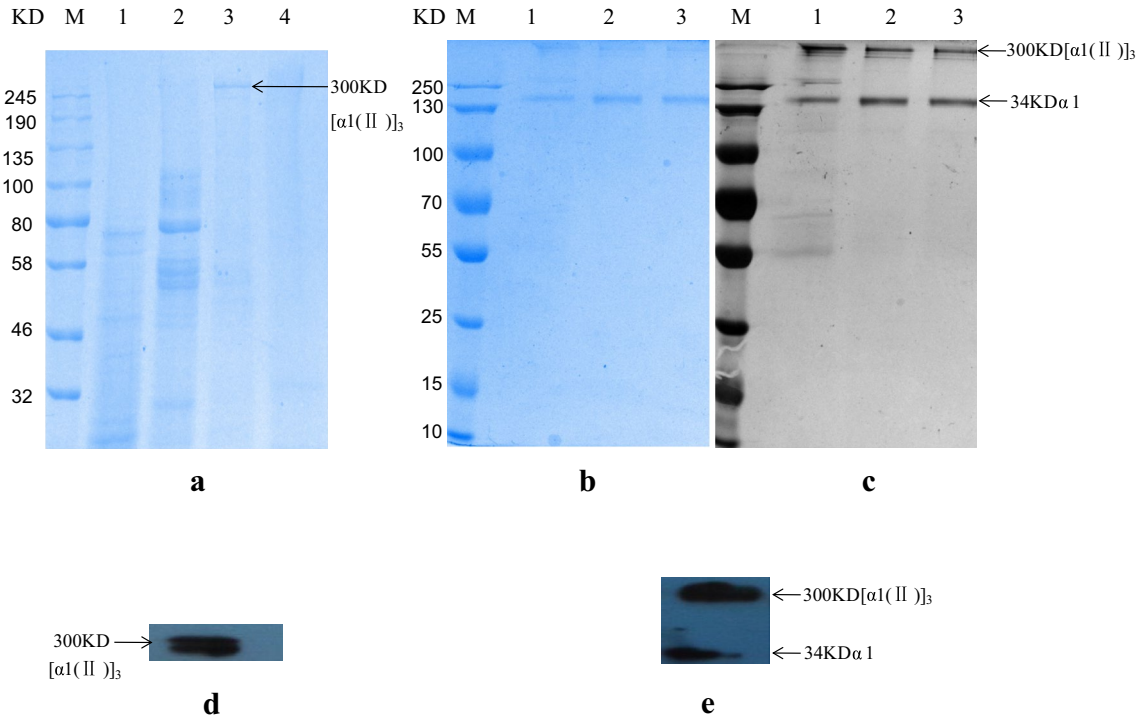

Fig. 4 SDS-PAGE and western blot analysis of purified recombinant human type II collagen. **a** Purification of recombinant human type II collagen from *Sf*9 cells on 8 % SDS-PAGE. *Lane M* color protein standard, broad range. *Lane 1* PB buffer washed Ni–NTA affinity column when the *Sf*9 cells sample flowed through the column; *lane 2* 50-mM imidazole elution buffer; *lane 3* 150-mM imidazole elution buffer; *lane 4* 0.5-M NaOH buffer. The $[\alpha1(II)]_3$ was labelled. **b**, **c** Purification of recombinant human type II collagen from silkworm larvae skin on 8 % SDS-PAGE with Coomassie staining (**b**) and silver staining (**c**). *Lane M* standard protein marker (10–250 KD). *Lane 1–4* were 150-mM imidazole elution buffer. The α band and $[\alpha1(II)]_3$ were labelled. **d** Western blot analysis of the putrefied recombinant human type II collagen from *Sf*9 cells infected with recombinant virus. **e** Western blot analysis of the purified proteins from the skin of infected silkworm larvae. The α band and $[\alpha1(II)]_3$ were labelled

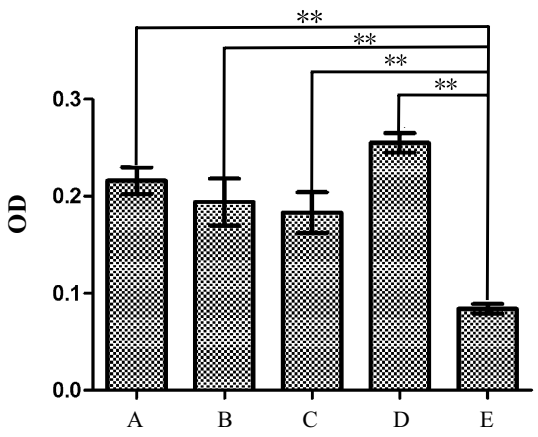

Fig. 5 Activity detection of recombinant human type II collagen expressed by silkworm larvae. Experimental cells: alb/c 3T3 cells; 570-nm absorbance, *lane A* 0.6-μM purified sample (recombinant human type II collagen expressed by silkworm larvae), OD = 0.211; *lane B* 0.4-μM purified sample, OD = 0.194; *lane C* 0.2-μM purified sample, OD = 0.183; *lane D* 0.1-μM bovine fibronectin (a positive control), OD = 0.268; *lane E* normal saline (a negative control), OD = 0.084. $^{**}P < 0.01$

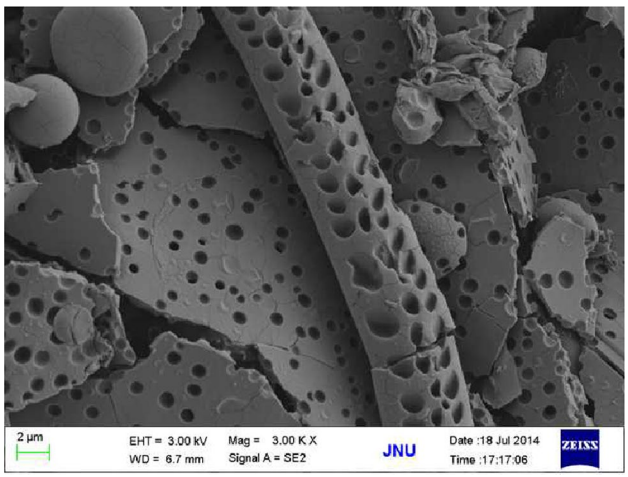

Fig. 6 SEM image of human type II collagen collected from silkworm larvae' skin. Scanning electron microscope (SEM) results show that the samples have the randomly distributed and pitted structures. *Bar* 2 μm

Western blotting results showed that the expression products of human type II collagen genes in both *Sf*9 cells and silkworm larvae lead to 300-kDa bands, which indicate collagen α-chains organized in a triple-helical structure. After purified by nickel column, the lyophilized type II collagen sample was analyzed by 8 % SDS-PAGE and western blotting. Subsequent analysis revealed the dissociation of the triple-helical arrangement of α-chains, thereby indicating that the triple-helical arrangement in the expressed collagen is unstable. Different types of collagens contain variations in α peptides and are characterized by multiple molecular subunits and super molecular structural arrangements. Because of these characteristics, the collagen family has many different members. However, collagen has the same basic component: the amino-acid sequence contains a Gly-X-Y repeat-X being proline (Pro) and Y being hydroxyproline (Hy-Pro) or hydroxylysine (Hy-Lys). These repeat sequences are largely responsible for the triple-helical characteristics of collagen and are highly utilized in biochemical modifications that give rise to the unique physical qualities of collagen. In collagen, the content of proline and lysine is particularly abundant, which is key in the formation of the triple-helical structure of α-chains. Proline-4-hydroxylase (P4H) acts on proline residues in the collagen primary structure to form hydroxyproline, which plays an important role in stabilizing the triple-helical structure of collagen through its facilitation of intermolecular hydrogen bonds. Hydroxylysine plays a similar role to hydroxyproline in this regard. It is worth nothing that in this particular study, human type II collagen was expressed in the absence of P4H and its lysine counterpart, thereby accounting for the lack of hydroxylation in our residues, which explains the ease of dissociation of the collagen triple-helical structure in our subsequent analyses. Analysis of the expression of recombinant human type II collagen in silkworm larvae by 8 % SDS-PAGE and western blotting demonstrated both silkworm haemolymph and skin expressed recombinant human type II collagen. However, target product purification in haemolymph was difficult due to the limited amount of haemolymph that can be extracted from each silkworm, the fact that haemolymph readily undergoes oxidation and, furthermore, is pigmented. In contrast, silkworm skin is easy to collect, as a larger quantity of skin can be collected per silkworm compared with haemolymph. Accordingly, silkworm skin was chosen for the extraction and purification of target protein. An assay of recombinant human type II collagen expressed by silkworm larvae demonstrated that the recombinant protein has considerable bioactive properties. At the same time, scanning electron microscopy (SEM) of our purified proteins revealed randomly distributed, pitted structures clearly. This was consistent with our expectations and results from other collaborators. Because we used the baculovirus expression system to express the recombinant collagen, as opposed to extracting collagen from fresh tissue samples, and because of our sample's aforementioned triple-helical instability, we did not observe the triple-helical motifs in native collagen. On conclusion, the baculovirus expression system is well suited for large gene/large molecular expressions. With respect to using the system to express human type II collagen, some problems still remain. Namely, the maintenance of the human type II collagen triple-helical structure greatly depends on the hydroxylation of proline and lysine within the Gly-X-Y motif. As noted, this current study did not utilize the hydroxylation enzyme P4H. Regardless, our system was still able to produce triple-helical collagen, albeit it was comparatively unstable and dissociated during further analysis into individual chains. Other studies have successfully expressed P4H with human type II collagen in insect cell lines (Nokelainen et al. 1998), so we speculate that P4H can similarly be co-expressed with the human type II collagen gene in our system, thereby lending greater stability to the triple-helical complex. All in all, the baculovirus-silkworm expression system shows promise in its relevance to the expression of high volume, large molecular complexes for both research and industrial purposes.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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