Production of a Non-Triple Helical Collagen α1 Chain in Transgenic Silkworms and Its Evaluation as a Gelatin Substitute for Cell Culture

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ABSTRACT: We generated transgenic silkworms that synthesized human type I collagen α1 chain [α1(I) chain] in the middle silk glands and secreted it into cocoons. The initial content of the recombinant α1(I) chain in the cocoons of the transgenic silkworms was 0.8%. The IE1 gene, a trans-activator from the baculovirus, was introduced into the transgenic silkworm to increase the content of the chain. We also generated silkworms homozygous for the transgenes. These manipulations increased the α1(I) chain content to 8.0% (4.24 mg per cocoon). The α1(I) chain was extracted and purified from the cocoons using a very simple method. The α1(I) chain contained no hydroxyprolines due to the absence of prolyl-hydroxylase activity in the silk glands. Circular dichroism analysis showed that the secondary structure of the α1(I) chain is similar to that of denatured type I collagen, demonstrating the absence of the triple helical structure. Human skin fibroblasts were seeded on the α1(I) chain-coated dishes. The cells attached and spread, although at decreased chain concentrations the spreading rate was lower than that of the collagen and gelatin. Cynomolgus monkey embryonic stem cells cultured on the α1(I) chain-coated dishes maintained an undifferentiated state after 30 passages, and their pluripotency was confirmed by teratoma formation in severe combined immunodeficient mice. These results show that the recombinant human α1(I) chain is a promising candidate biomaterial as a high-quality and safe gelatin substitute for cell culture.

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KEYWORDS: transgenic silkworm; cocoon; collagen; gelatin; cell culture

Introduction

Collagen, a protein with a triple helical structure composed of three α chains, is used as a biomaterial for a variety of medical and cosmetic applications (Lee et al., 2001; Miyata et al., 1992). Gelatin, the denatured and fragmented form of collagen, is applied in medicine as a material for oral drug delivery and parenteral formulations (Gill and Feinberg, 2001; Tabata and Ikada, 1998). These materials come mainly from animal sources, which carry a risk of pathogen contamination and can also cause allergic reactions (Bradley, 1993; Mullins et al., 1996). One promising approach to overcome such problems is to produce them by recombinant means using appropriate hosts. For example, collagens have been produced successfully using host cells possessing prolyl-hydroxylase activity, which is required for the formation of the triple helical structure (Berg and Prockop, 1973; Fichard et al., 1997; Geddis and Prockop, 1993). Collagens have also been produced in host cells that did not possess sufficient prolyl-hydroxylase activity for triple helix formation but were transfected with prolyl-hydroxylase genes (Olsen et al., 2003; Werten et al., 1999).

We previously generated transgenic silkworms that synthesized a recombinant fusion protein containing the collagen sequence in their silk glands and secreted it into cocoons. Due to the absence of prolyl-hydroxylase activity in the glands, prolyl residues in the collagen sequence were not hydroxylated (Tomita et al., 2003, 2005). To produce
prolyl-hydroxylated protein, prolyl-hydroxylase genes were simultaneously introduced to the silkworms along with the recombinant protein gene. The resulting silkworms produced a protein containing a prolyl-hydroxylated collagen sequence in their cocoons (Adachi et al., 2006). While these studies demonstrated the possibility of mass production of recombinant collagens in transgenic silkworms, we recognized an important issue that needed to be addressed: given that the recombinant protein was incorporated into insoluble silk fibers, the protein could not be extracted without using strong chaotropic reagents.

Silk fibers are composed mainly of two types of protein: fibroin and sericin. The former comprises 70–80% of all silk proteins, constitutes the core of insoluble silk fibers, and is synthesized in the posterior silk glands (PSGs). Sericin, which accounts for 20–30% of silk proteins, refers to a group of hydrophilic glue proteins that surround the fibroin core and are synthesized in the middle silk glands (MSGs; Garel et al., 1997; Grzelak, 1995). The above-described recombinant fusion protein containing the collagen sequence was expressed in the PSGs, resulting in production of the recombinant protein into the insoluble fibroin core (Tomita et al., 2005). Recently, we also developed a sericin promoter-driven recombinant protein expression system, in which recombinant proteins are expressed in the MSGs and secreted into hydrophilic sericin layers of silk fibers. Green fluorescent protein (Tomita et al., 2007), human serum albumin (Ogawa et al., 2007), and mouse IgG (Iizuka et al., 2005) were successfully produced in cocoons. These proteins were extractable from cocoons by soaking them in mild neutral aqueous solutions such as phosphate-buffered saline (PBS) or Tris-buffered saline.

In this study, we generated transgenic silkworms that expressed the full-length triple helical region of the human type I collagen α1(1) chain in the MSGs. Type I collagen is the most abundant fibril-forming collagen in the human body. A common form of type I collagen is a heterotrimer composed of two α1(1) chains and one α2(1) chain. It is also known that α1(1) chains are able to form a homotrimer in the absence of α2(1) chain (Nicholls et al., 1979). The recombinant α1(1) chain expressed in the MSGs was efficiently secreted into cocoons and was easily recoverable. Given that the silk glands had no prolyl-hydroxylase activity, the chains contained no hydroxyproline residues. In addition, the recombinant α1(1) chain expressed in this study lacks the telopeptide and propeptide promoting triple helix formation (Doeger and Fessler, 1986; Olsen et al., 2001; Rosenbloom et al., 1976). Therefore, the α1(1) chains did not form the homotrimer with the triple helical structure, and their physio-chemical properties were similar to those of gelatin rather than collagen. We tested the possibility of using the recombinant α1(1) chain as a cell culture scaffold, and found that cells could be cultured on the chain as on marketed gelatin. As the recombinant α1(1) chain has uniform molecular weight and no risks of animal-derived pathogen contamination, the recombinant chain may be useful as a high-quality and safe substitute for marketed gelatin.

Materials and Methods

Experimental Animals

A strain of silkworm, pnd-w1, was obtained from the National Institute of Agrobiological Science (Tsukuba, Japan). Larvae were reared at 25°C on an artificial diet (Nusan Corporation, Yokohama, Japan).

Vector Construction for Transgenic Silkworms

The vector COL1A1/pMSG for transgenic silkworms was constructed using a full-length cDNA coding for the pro α1 chain of human type I procollagen (GenBank Accession No. Z74615) obtained by RT-PCR from human placenta total RNA (Clontech, Palo Alto, CA). The PCR-amplified product was cloned into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA) using a TOPO cloning system (Invitrogen), which yielded pCR4/COL1A1. To obtain the DNA fragment containing the baculovirus polyhedrin 5’-untranslated region (UTR; nt 140–189; GenBank Accession No. M30925; Iizuka et al., 2008), the signal sequence of human calreticulin (nt 109–159; GenBank Accession No. M84739), and the cDNA for the triple helical region of the α1(1) chain (nt 654–3695; Fig. 1A), PCR was performed using pCR4/COL1A1 as a template. A set of primers, calSP/ COL1A1-F (5’-ATGCTGCTATCCGCGTGGTGCTGCTCG- GCCCTCCTGGCCGCTGGCGGCCCACATGGTTCCCTC- CT3’) and NruI/UTR-COL1A1-R (5’-TGCGGAGCTCGGCGCGACC- GGACC3’) was used for the first PCR. A second PCR was performed using the product of the first PCR as a template and NruI/Bm5UTR/calSP-F (5’-TCGCGAAGATTTTACTGGTACAGTTTTTGTAAT- TAAAACCTATAATATATGCCGCTGGC3’) and NruI/UTR-COL1A1-R as a primer set. The amplified product was cloned into the pCR4-TOPO vector to generate pCR4/BmUTRCOLSP/COL1A1. The DNA fragment was re-excised from pCR4/BmUTRCOLSP/COL1A1 by digestion with NruI, and inserted between the Bombyx mori sericin 1 promoter and the fibroin light chain 3’-flanking sequence of pMSG1.1MG (Iizuka et al., 2009), giving rise to COL1A1/pMSG (Fig. 1B).

A vector carrying the gene of baculovirus trans-activator IE1 (Tomita et al., 2007) was prepared as follows. To obtain the DNA fragment consisting of the polyhedrin 5’-UTR and the IE1 gene, PCR was performed using pIE1 (Tomita et al., 2007) as a template. A set of primers, EcoRV/Bm5UTR/IE1-F (5’-GATATCAGATTTTACGTGGCGCTGTCG- GTAACAGTTTTGTAATAAAAACCTATAATATGACGCAAATTAATTTTACGCGTGCG-3’) and Bgl/IE1-R (5’- AGATCTTTATTTAAACCTATAATATTTACGCGTGCG-3’), was used for the PCR. The amplified product

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pIM1 was injected into eggs, and transgenic silkworms were created as described above, except that screening was performed by observing MGFP fluorescence in the larval body. The resulting transgenic silkworm (IM1) was crossed with silkworms carrying the α1(I) chain to obtain silkworms hemizygous for both the IE1 and the α1(I) chain genes.

To generate silkworms homozygous for the IE1 and the α1(I) chain genes, the above hemizygous silkworms were crossed with one another. From the next generations, homozygous silkworms for both genes were screened by genomic PCR using DNA extracted from blood cells as a template. Primers used in this PCR were designed from the genomic sequences flanking the transgene insertions, which were determined with an APA Transgene Locator Kit (BIO S&T Inc., Montreal, QC, Canada) according to the manufacturer’s instructions.

**SDS–PAGE and Western Blotting**

Proteins in the sericin layer of silk fibers were extracted by incubating cocoons at 80°C for 5 min in a buffer consisting of 8 M urea, 2% 2-mercaptoethanol, and 50 mM Tris–HCl, pH 8.0. After centrifugation at 23,500 g for 5 min, the obtained supernatant was electrophoresed on 0.1% SDS–20% polyacrylamide gradient gels (Atto, Tokyo, Japan) in running buffer (0.1% SDS, 12.5 mM Tris, and 125 mM glycine). The gels were stained with CBB–R250, and densitometric analyses were performed using the image-processing program, ImageJ (http://rsb.info.nih.gov/ij/). For Western blotting, the proteins on the gels were transferred onto PVDF membranes (Immobilon-P; Millipore, Billerica, MA), reacted with antihuman/bovine type I collagen antibodies (LB-1190; Cosmo Bio, Tokyo, Japan) and then with horseradish peroxidase (HRP)-linked anti-(rabbit IgG) antibodies (Cell Signaling Technology, Danvers, MA). The antigen–antibody complexes were visualized using the ECL Western Blotting Detection System (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

**Purification of the Recombinant α1(I) Chain From Transgenic Silkworm Cocoons**

Cocoons were crushed into powder using a mill, suspended in a solution of 8 M urea and 0.5 M CH₃COOH, pH 2.0, at a concentration of 10 mg powdered cocoons/mL, and incubated at 4°C for 2 days with stirring. The resulting solution was filtered through filter paper and 70-μm nylon mesh. The solution was concentrated approximately threefold with an ultrafiltration membrane (Millipore) and urea was removed from the solution by adding 0.5 M CH₃COOH, pH 2.0, and subsequent ultrafiltration. NaCl was then added to the solution at a concentration of 0.5 M, and precipitated sericin was removed by centrifugation. Next, the recombinant α1(I) chain in the solution was collected by the addition of 3.5 M NaCl. The precipitate was

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**Figure 1.** The structure of the transformation vector. A. The structure of the amino acid sequence of the α1(I) chain. The sequence of the α1(I) chain consists of a 17 amino acid (AA)-long calreticulin signal peptide (open box) and a 1,014 AA-long triple helical region of the α1(I) chain (filled box). B. The structure of the transformation vector COL1A1/pMSG. COL1A1/pMSG contained two expression units between the right and left arms of piggyBac as follows: 3xP3 promoter-driven MGFP cDNA with the SV40 polyA signal sequence (SV40 polyA) and the baculovirus-derived enhancer hr3-linked sericin1 promoter (ser1) promoter-driven α1(I) chain cDNA with the fibroin light chain gene polyA signal sequence (fibL 3′-flanking). ITR, inverted terminal repeat. C. Transformation vector pIM1. pIM1 contained two expression units between the right and left arms of piggyBac as follows: Bombyx mori A3 actin promoter-driven MGFP cDNA with the SV40 polyA and ser1 promoter-driven ie1 gene with the fibL 3′-flanking.

**Generation of Transgenic Silkworms**

COL1A1/pMSG was injected with the helper vector pHA3pIG (Tamura et al., 2000) into pre-blastoderm embryos as described previously (Tomita et al., 2003). Hatched G₀ larvae were reared at 25°C to the moth stage. G₁ embryos obtained by sibling mating were screened for the expression of Monster Green Fluorescent Protein (MGFP; Promega, San Luis Obispo, CA) in the eyes and nervous tissues to obtain transgenic silkworms.
solubilized in 0.5 M CH₃COOH and subjected to dialysis against water.

### N-Terminal Sequencing and Measurement of Amino Acid Composition

The N-terminal sequences of the purified α1(I) chain were determined with a G1000A protein sequencer (Hewlett Packard, Palo Alto, CA) using the standard protocol of Edman degradation. For the degradation reaction, 36 pmol of the chain were loaded onto the sequencer and the reaction was carried out for five cycles.

The purified α1(I) chain was hydrolyzed in 6 N HCl for 22 h at 110°C under vacuum, and analyzed for amino acid composition using a Hitachi 835 amino acid analyzer (Hitachi, Tokyo, Japan).

### Determination of Melting and Gelation Points

The purified α1(I) chain was dissolved in distilled water at a concentration of 50 mg/mL, and the solution was subjected to determination of gelation and melting points as follows. The α1(I) chain solution was gradually cooled from 35 to −5°C at a rate of 1°C/min with a thermal cycler (Atto). The gelation point was determined by reading the temperature of the sample when its fluidity disappeared. For the analysis of the melting point, the α1(j) chain solution was incubated in icy water for 30 min to create a gel. The α1(I) chain gel was gradually heated from 0 to 40°C at a rate of 1°C/min with the thermal cycler. The melting point was determined by reading the temperature of the sample when the bubble at the bottom of the tube reached the surface of the sample solution.

### Measurement of Circular Dichroism (CD) Spectra

CD spectra were recorded for the recombinant α1(I) chain using a spectropolarimeter J-820 (Jasco, Tokyo, Japan). The purified α1(I) chain was dissolved in 50 mM CH₃COOH at a concentration of 100 μg/mL for measurement in far ultraviolet (190–240 nm) regions, and the solution was placed in a cuvette with 2-mm pass length. The temperature was kept at 4°C during the procedures. Measurements were performed three times and the averaged values were plotted. Thermal transition curves were recorded by measuring molar ellipticity at 224 nm between 5 and 60°C at a rate of 30°C/h. The denaturing temperature was calculated with J-820 software (Jasco).

### Endotoxin Test

An endotoxin level of the recombinant α1(I) chain was quantified using the limulus amebocyte lysate (LAL) as per the manufacturer’s instructions (Endospecy ES-50M and Toxicolor DIA-MP; Seikagaku Biobusiness, Tokyo, Japan).

Briefly, 50 μL standards or samples diluted with endotoxin-free water were mixed with LAL and incubated at 37°C for 30 min. After the substrate solution was added, the absorbance at 545 nm was measured. A standard curve was constructed by using the standards in the range of 0.02–0.1 EU/mL, and the concentration of endotoxin in each sample was determined by plotting the absorbance to the standard curve.

### Quantifying the Spread of Human Dermal Skin Fibroblasts

The cell adhesivity to the recombinant α1(I) chain was analyzed by a quantitative cell-spreading assay using human dermal skin fibroblasts (HSFs; Kurabo, Osaka, Japan) as described previously (Yamada and Kennedy, 1984). In brief, a 96-well tissue culture plate (Becton Dickinson Labware, Franklin Lakes, NJ) was incubated with the recombinant α1(I) chain at various concentrations (0.31, 0.62, 1.25, 2.5, and 5.0 μg/mL) at 37°C for 1 h, treated with heat-denatured 0.5% bovine serum albumin to block the direct interaction between cells and the plate surface, and extensively washed with PBS. HSFs were seeded on the wells at a density of 1 × 10⁴ cells per well, cultured in serum-free Dulbecco’s modified Eagle’s medium for 1 h, fixed in 4% paraformaldehyde, and viewed through a phase-contrast microscope (Nikon, Tokyo, Japan) to calculate the ratio of fully spreading cells to all cells observed in five randomly selected fields. HSFs were also cultured on the 96-well tissue culture plate coated with 10 μg/mL of the α1(I) chain as above to observe the cell morphology.

### Culture of Cynomolgus Monkey Embryonic Stem (ES) Cells on Dishes Coated With the Recombinant α1(I) Chain

Cynomolgus monkey ES cells were cultured on murine embryonic fibroblast (MEF) feeder cells in Petri dishes (90 mm in diameter) according to previously established protocols (Cameron et al., 2006). Briefly, dishes were coated with the recombinant α1(I) chain by incubation with a solution of 1 mg/mL α1(I) chain for 30 min at room temperature. γ-Irradiated MEF cells were then cultured on the coated dishes as feeder cells for 1 day. Subsequently, monkey ES cells were seeded on MEF feeder layers and maintained by changing the medium. Immunostaining of monkey ES cell colonies was performed using NANOG, TRA1-81, SSEA-4, SOX2, and OCT4-specific primary antibodies (Millipore) according to the manufacturer’s protocol. Nuclei were visualized by DAPI staining (Lin et al., 1976).

Monkey ES cells were injected subcutaneously into the hind leg of severe combined immunodeficient (SCID) mice (Suemori et al., 2001). Two months after injection, the mice were killed to remove teratomas. The teratomas...
were then fixed with 4% paraformaldehyde in PBS, paraffin-embedded, sectioned, and histologically analyzed following staining with hematoxylin and eosin.

**Results**

**Generation of Transgenic Silkworms**

COL1A1/pMSCG was injected into 9,834 pre-blastoderm embryos, and the hatched 5,282 G₀ larvae were allowed to develop to moths. G₁ embryos from the G₀ moths were screened for MGFP expression in the eyes and nervous tissues to obtain transgenic silkworms. Genomic Southern blot analysis of the transgenic silkworms demonstrated the existence of 41 independent transgenic lines with respect to the chromosomal insertion positions and copy numbers of the transgenes. Among them, 34 lines of transgenic silkworms with a single-copy transgene were selected, and the cocoon proteins of the lines were analyzed by SDS–PAGE. The line with the highest level of α1(I) chain expression was crossed with wild-type silkworms, and offspring hemizygous for the α1(I) chain gene were used in the following experiments as the COL249 line.

The worms of the COL249 line spun cocoons that were normal in appearance, size, and weight. Proteins in the sericin layer of the silk fibers of COL249 and wild-type silkworms were separated by SDS–PAGE and stained with CBB (Fig. 2, lanes 1–2). A band with an apparent molecular weight of 120 kDa was seen only in the cocoon proteins of COL249 (Fig. 2, lane 1). This band reacted with antimouse/bovine type I collagen antibody (Fig. 2, lane 6), indicating that this was the recombinant product from the human α1(I) chain gene. The band intensity of the recombinant α1(I) chain on the CBB-stained gel was quantified by densitometry. The content of the α1(I) chain was estimated to be 0.8% of the dried cocoon.

To increase the α1(I) chain content in the cocoon, a COL249 moth was crossed with an IM1 moth bearing the gene of the baculovirus-derived trans-activator IE1. Approximately 25% of the offspring carried both the α1(I) chain and IE1 genes hemizygously. The α1(I) chain/IE1 hemizygous silkworms (COL249/IM1) were further crossed with each other and silkworms homozygous for both the α1(I) chain and IE1 genes (COL249/IM1²) were screened from the offspring by genomic PCR. Proteins in the cocoon extracts of COL249, COL249/IM, and (COL249/IM)² were separated by SDS–PAGE and stained with CBB (Fig. 3). By measuring the band intensity, the contents of the α1(I) chain in cocoons of the silkworm lines COL249, COL249/IM, and (COL249/IM)² were estimated to be 0.8%, 4.8%, and 8.0%, respectively. The average weights of cocoons in the COL249, COL249/IM, and (COL249/IM)² lines were 72, 65, and 53 mg, respectively. Although the cocoon weight decreased slightly with increased transgene copy numbers, the synthesis of the recombinant α1(I) chain per larvae was improved by this procedure; the amounts of the chain per cocoon of the COL249, COL249/IM, and (COL249/IM)² lines were 0.58, 3.12, and 4.24 mg, respectively.

**Extraction and Purification of the Recombinant α1(I) Chain From Cocoons**

The extraction efficiency of the recombinant α1(I) chain from cocoons was examined. The powder of (COL249/IM)² cocoons was suspended in either PBS (Fig. 4A, lane 3), 0.5 M CH₃COOH, pH 3.0 (Fig. 4A, lane 4), 0.5 M CH₃COOH, pH 2.0 (Fig. 4A, lane 5), or 8 M urea and 0.5 M CH₃COOH, pH 2.0 (Fig. 4A, lane 6), at 4°C for 16 h, and the extracted proteins were analyzed by SDS–PAGE. Total proteins in the sericin layer of silk fibers of wild-type and transgenic silkworms were extracted by incubating cocoons at 80°C for 5 min in a buffer consisting of 8 M urea, 2% 2-mercaptoethanol, and 50 mM Tris–HCl, pH 8.0, separated by SDS–PAGE, and stained with CBB (left panel). Aliquots of the cocoon extracts were also assessed by Western blotting with antimouse/bovine type I collagen antibodies (right panel). Bovine pepsinized type I collagen in the amounts indicated was analyzed by CBB staining and Western blotting as a standard (std). The arrowheads in lanes 1 and 6 point to the band of the recombinant α1(I) chain. The arrows in lane 5 point to α1(I) and α2(I) chains of bovine type I collagen. Arabic numerals at the left side are molecular masses in kDa.
cocoons, and a major part of the \( \alpha_1(I) \) chain was extractive with 8 M urea and 0.5 M CH\(_3\)COOH, pH 2.0.

For purification of the \( \alpha_1(I) \) chain, 30 g of (COL249/IM\(^2 \)) cocoon powder, which was estimated to contain 2.4 g of the \( \alpha_1(I) \) chain, were suspended in 8 M urea and 0.5 M CH\(_3\)COOH, pH 2.0. The extracted \( \alpha_1(I) \) chain (Fig. 4B, lane 2) was concentrated by ultrafiltration (Fig. 4B, lane 3). The urea in the solution was removed by adding 0.5 M CH\(_3\)COOH, pH 2.0, and subsequent ultrafiltration. In this process, the endogenous sericin in the extract formed an insoluble aggregate, increasing the \( \alpha_1(I) \) chain content in the extract (Fig. 4B, lane 4). Small amounts of contaminated sericin were removed by 0.5 M NaCl precipitation (Fig. 4B, lane 5), and the \( \alpha_1(I) \) chain in the supernatant was then collected by precipitation with 4 M NaCl. The collected \( \alpha_1(I) \) chain was dissolved in 0.5 M CH\(_3\)COOH, pH 2.0, again, and the \( \alpha_1(I) \) chain solution was finally dialyzed against water (Fig. 4B, lane 6). The proteins in each
puriﬁcation step and total proteins in the sericin layer (Fig. 4B, lane 1) were analyzed by SDS–PAGE, demonstrating that this simple puriﬁcation process is sufﬁcient to purify the α1(I) chain to apparent homogeneity. As a result, 990 mg of the α1(I) chain were puriﬁed from 30 g of cocoons; the recovery rate was estimated to be approximately 41%.

Biochemical Characterization

The puriﬁed recombinant α1(I) chain was analyzed by SDS–PAGE. Although small amounts of degradation products were found, the puriﬁed recombinant chain was composed of the polypeptide with a uniform molecular weight. The molecular weight of the chain was slightly smaller than the standard bovine α1(I) chain (Fig. 4C, lanes 1 and 2), indicating the possibility of insuﬃcient prolyl-hydroxylation in the recombinant chain. The dimer (β chain) and trimer (γ chain) of the α chain, which were present in the standard collagen, were not detected from the puriﬁed recombinant chain, suggesting the absence of covalent cross-linking among the α1(I) chains. The molecular weight distribution of the recombinant α chain was quite diﬀerent from that of the alkali-treated bovine (Fig. 4C, lane 3) or acid-treated porcine gelatins (Fig. 4C, lane 4). The gelatins gave broad molecular weight distributions because they were hydrolyzed products of collagens.

The α1(I) chain was subjected to an amino acid sequencer with ﬁve cycles of Edman degradation. The N-terminal amino acid sequencing of the α1(I) chain detected major and minor amino acid peaks in each cycle as shown in Table I. The sequence deduced from the minor peaks (GPM) was consistent with that of the predicted signal peptide cleavage (Fig. 1A) although peaks were not detected in the fourth and ﬁfth cycles. The sequence from the major peaks (MGPSG) was probably derived from a cleavage at two amino acids downstream of the predicted site.

The amino acid composition of the puriﬁed α1(I) chain was determined after acid hydrolysis using a Hitachi L835 automated analyzer (Table II). The determined values were very similar to the predicted ones, except for the absence of hydroxyprolines and hydroxylsines.

The endotoxin levels of the α1(I) chain and the porcine gelatin were measured. The endotoxin level of the α1(I) chain was much lower (26 EU/g) than the gelatin (6,400 EU/g).

Structural Characterization

Far-ultraviolet (190–240 nm) CD spectra were recorded for the recombinant α1(I) chain, the native bovine type I collagen, the heat-denatured bovine type I collagen, and the porcine gelatin (Fig. 5A). The positive peak at 224 nm that is characteristic of the triple helical structure of collagen (Miller and Gay, 1982) was observed in the type I collagen. The gelatin exhibited a positive low peak at this wavelength, suggesting that the gelatin contained a partly formed triple helical structure in the molecule. In contrast, the recombinant α1(I) chain did not show a positive peak at 224 nm. The peak of the type I collagen disappeared when it was heat-denatured. The spectra of the denatured collagen were almost identical to those of the recombinant α1(I) chain. These results suggest that the α1(I) chain contained no triple helical structure. A negative peak at 198 nm represents the triple helical structure (Miller and Gay, 1982). The peak intensity at this wavelength of the recombinant α1(I) chain was similar to that of the heat-denatured collagen rather than that of gelatin, conﬁrming the absence of the triple helical structure in the recombinant chain.

The 224-nm spectra were recorded for the recombinant chain, the native collagen, the denatured collagen and the gelatin at temperatures from 4 to 60°C (Fig. 5B). Apparent structural transition of the native collagen was observed in the range 39–46°C, which is in accordance with a report that the denatured temperature of bovine type I collagen is 42.8°C (Peltonen et al., 1980). In contrast, the recombinant α1(I) chain, the denatured collagen and the gelatin showed slight structural changes in the range 25–45°C.

The melting and gelation points of the 5% α1(I) chain or the gelatin solution were measured as described in the Materials and Methods Section. The melting and gelation

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<th>Recombinant α1(I) chain</th>
<th>Human α1(I) chain (predicted)</th>
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<td>Aspartic acid</td>
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<td>Tryptophan</td>
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<td>0.00</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>0.00</td>
<td>11.44*</td>
</tr>
<tr>
<td>Proline</td>
<td>22.80</td>
<td>11.83</td>
</tr>
<tr>
<td>Total</td>
<td>100.00</td>
<td>100.00</td>
</tr>
</tbody>
</table>

*Assuming that all of lysine and proline residues in Y-position are hydroxylated.

Table I. N-terminal sequencing.

<table>
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<th>Amino acid number</th>
<th>1</th>
<th>2</th>
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<tr>
<td>Major peaks</td>
<td>M</td>
<td>G</td>
<td>P</td>
<td>S</td>
<td>G</td>
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<tr>
<td>Minor peaks</td>
<td>G</td>
<td>P</td>
<td>M</td>
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</tbody>
</table>
points of the α1(I) chain were 17 and 10°C, respectively, while the melting and gelation points of the gelatin were 30 and 26°C, respectively. Thus, both the melting and gelation points of the α1(I) chain were lower than the respective points of the gelatin, which may support the result from the measurement of CD spectra showing an absence of triple helical structures in the α1(I) chain. We also analyzed whether the α1(I) chain formed collagen fibrils under the physiological conditions as native collagen (Michalopoulos and Pitot, 1975) and found that the α1(I) chain did not form the fibrils (data not shown).

Cell Biological Properties

To investigate the cell biological properties of the recombinant α1(I) chain, HSFs were cultured on dishes coated with the α1(I) chain, the native collagen, or gelatin at various concentrations, and cell spreading on the materials was analyzed as described in the Materials and Methods Section (Fig. 6A). HSFs spread on all coating materials in a concentration-dependent manner. On collagen at concentrations of more than 0.63 µg/mL, HSFs spread at a rate of 100%. More than 80% of the cells spread on gelatin at concentrations >0.63 µg/mL, but 100% cell spreading was never observed even at the highest concentration tested (5.0 µg/mL). Cell spreading rates for the α1(I) chain at concentrations of <2.5 µg/mL were slightly lower compared to those on gelatin at the same concentrations. However, HSFs spread on the α1(I) chain at a similar rate to on gelatin when inoculated at a concentration of 5.0 µg/mL. No differences were observed among cell morphologies when the cells were cultured on the α1(I) chain, the native collagen, or the gelatin at a concentration of 10 µg/mL (Fig. 6B, panels a–c). Cell-spreading was not observed on the uncoated dishes (Fig. 6B, panel d).

Cynomolgus monkey ES cells were cultured on feeder cells that had been cultured on dishes coated with the α1(I) chain or porcine gelatin. The ES cells cultured on dishes coated with the α1(I) chain formed tightly packed and flattened colonies (Fig. 7A, panel a). This morphology was the same as that of ES cell colonies cultured on dishes coated with porcine gelatin (Fig. 7A, panel b). Immunocytochemical studies confirmed that the monkey ES cell colonies on the α1(I) chain expressed the ES cell marker proteins NANOG, TRA1-81, SSEA-4, SOX2, and OCT4 (Fig. 7B). When the ES cells were subcutaneously injected into SCID mice after the passages on the α1(I) chain, the cells formed teratomas in the mouse tissues. Histological analyses of the teratomas showed formation of pigment epithelium, gastrointestinal epithelium, and cartilage (Fig. 7C). Thus, the α1(I) chain was confirmed to be useful for the maintenance of monkey ES cells.

Discussion

We generated transgenic silkworms that secreted the recombinant human α1(I) chain into the sericin layer of silk fibers. The content of the α1(I) chain in the cocoons of the established line COL249 was estimated to be 0.8%. By introducing the gene of the trans-activator IE1 into the silkworm as in our previous studies (Ogawa et al., 2007; Tomita et al., 2007), the expression of the α1(I) chain was enhanced to 4.8%. We then generated silkworms (COL249/
IM)² homozygous for both the α1(I) chain and IE1 genes. This manipulation increased the α1(I) chain content up to 8.0%. Given that the average weight of a (COL249/IM)² cocoon was 53 mg, the α1(I) chain content per cocoon was calculated to be 4.24 mg. If 1,000 (COL249/IM)² silkworms were reared, we could produce 4.24 g of the α1(I) chain. The (COL249/IM)² silkworm was generated from the pnd-w1 strain, which produces small cocoons (50–70 mg). Our preliminary experiment revealed that the cocoon weight could be increased to approximately 150 mg by crossing it with typical silkworm strains that produce 300- to 500-mg cocoons, leading to elevation of the α1(I) content to more than 10 mg per cocoon (data not shown). We also demonstrated the superiority of the transgenic silkworm system for the purification of the recombinant α1(I) chain. The α1(I) chain was highly purified from the cocoon extract by a simple method consisting of ultrafiltration and salt precipitation. Thus, this study offers experimental evidence for the viability of using transgenic silkworms in the production of the human recombinant α1(I) chain on an industrial scale.

Our previous study demonstrated that prolyl-hydroxylase activity is absent in silk glands (Adachi et al., 2005), and the recombinant fusion protein containing the collagen sequence expressed in the glands includes no hydroxyprolines (Tomita et al., 2005). The recombinant α1(I) chain produced in this study also contained no hydroxyprolines as predicted. The presence of hydroxyprolines is a prerequisite for forming the stable collagen triple helix (Berg and Prockop, 1973). In addition, the α1(I) chain did not contain the C-telopeptide and C-propeptide, which are known to promote triple helix formation (Doege and Fessler, 1986; Rosenbloom et al., 1976). Therefore, we postulated that the α1(I) chain is not capable of forming the triple helix. Indeed, CD spectra of the chain showed a complete absence of the triple helical structure. The importance of the telopeptide and propeptide for the triple helix formation was also shown in the previous studies. Unhydroxylated type I collagen with the telopeptide, and unhydroxylated α1(I) chain with the telopeptide and propeptide were synthesized as correctly folded triple helices in yeast (Olsen et al., 2001) and tobacco (Ruggiero et al., 2000), respectively. On the other hand, this study revealed that the animal-derived gelatin contained a partially folded triple helix, suggesting the significance of hydroxyprolines in the stability of triple helix. To further clarify the difference of physiological properties among the recombinant α1(I) chain, the gelatin and the collagen, we investigated the gelation and fibril-forming properties of the α1(I) chain. Unlike the collagen, the α1(I) chain did not form collagen fibrils under the physiological conditions examined. In contrast, the α1(I) chain, as well as the gelatin, gelated at lower temperatures than physiological ones. The melting and gelation points of the α1(I) chain were lower than those of gelatin. Thus, the physico-chemical properties of the recombinant α1(I) chain were similar to gelatin rather than collagen. However, due to the complete absence of the triple helical structure, the properties of the α1(I) chain differed slightly from those of gelatin.

The α1(I) chain promoted cell attachment and the spread of HSFs, but the cell-spread rates for the α1(I) chain as well as gelatin were lower than those for collagen at all concentrations tested. At decreased concentrations of the materials, fewer cells spread on the α1(I) chain than on gelatin. Thus, HSFs were likely able to distinguish among these three materials. The cell–collagen interaction is mediated via integrins. Integrins α1β1 and α2β1 recognize collagens as collagen receptors (Hynes, 2002), and integrin

![Figure 6](image-url)

**Figure 6.** Spreading of HSFs on the α1(I) chain-coated dishes. A: Cell spreading assay using HSFs. The wells of tissue culture plates were coated with the α1(I) chain (black line), bovine type I collagen (dotted line), and porcine gelatin (dashed line) at various concentrations, and treated with heat-denatured bovine serum albumin to block the direct interaction between cells and the plate. HSFs were seeded on these wells and cultured for 1 h. The cells were then fixed, and the ratio of spreading cells to all cells in observed fields was calculated. B: Cell morphology of HSFs cultured on the α1(I) chain. HSFs were cultured on dishes coated with 10 μg/mL of the α1(I) chain (a), bovine type I collagen (b), porcine gelatin (c). The cells were also cultured on the uncoated but the albumin-treated dish (d). Scale bar, 10 μm.
α5β1 indirectly recognizes it via collagen-bound fibronectin (Mould et al., 1997). Although the detailed mechanism is unknown, the complete absence of hydroxyprolines or the triple helical structure in the α1(I) chain may be responsible for this discrimination. At concentrations >5.0 mg/mL, however, HSFs spread on the α1(I) chain at a similar rate to that of gelatin. At a concentration of 10 μg/mL, the cell morphology was indistinguishable from that on collagen or gelatin. These results emphasize the practical utility of the recombinant α1(I) chain as a cell scaffold.

To demonstrate the practicality of the α1(I) chain, monkey ES cells were cultured on chain-coated dishes. After 30 passages, the monkey ES cell colonies maintained excellent morphology and the expression of several marker proteins for ES cells. The pluripotency of the cells was also confirmed by the formation of teratomas in SCID mice. Sections of the teratomas that formed were stained with hematoxylin and eosin. Arrowheads in (a–c) point to the pigment epithelium, gastrointestinal epithelium, and cartilage, respectively. Scale bars, (a) and (b): 200 μm; (c): 1 mm.

Gelatins are generally used for culturing ES or iPS cells. However, most marketed gelatins are derived from bovine or porcine bone, and therefore there is a risk of contamination with animal-derived pathogens, including viruses. In contrast, the recombinant α1(I) chain developed in this study does not pose such a risk because the chain is extracted from silk cocoons without using animal-derived materials. In addition, the α1(I) chain is composed of human sequences with constant molecular weight. Unlike the animal-derived gelatin extracted by hydrolyzing tissue collagens, the quality of the chain can be easily controlled with lot-to-lot consistency. The endotoxin level of the α1(I) chain was much lower than marketed gelatins. The recombinant α1(I) chain is a promising candidate material for use as a high-quality gelatin substitute for tissue engineering, drug delivery, and other applications.

References
Adachi T, Tomita M, Yoshizato K. 2005. Synthesis of prolyl 4-hydroxylase α subunit and type IV collagen in hemocytic granular cells of silkworm,


