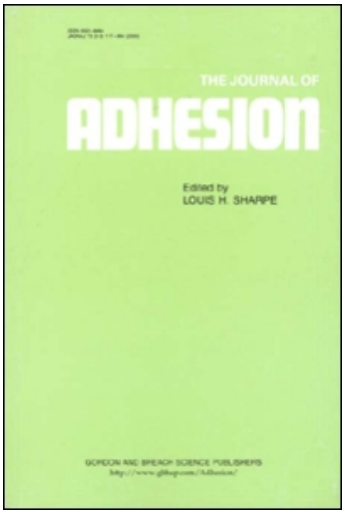

The Journal of Adhesion
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713453635

Towards the Recombinant Production of Mussel Byssal Collagens

Anja Hagenau[a]; Thomas Scheibel[a]
[a] Lehrstuhl für Biomaterialien, Universität Bayreuth, Bayreuth, Germany

Online publication date: 05 February 2010

To cite this Article Hagenau, Anja and Scheibel, Thomas(2010) 'Towards the Recombinant Production of Mussel Byssal Collagens', The Journal of Adhesion, 86: 1, 10 — 24
To link to this Article: DOI: 10.1080/00218460903417701
URL: http://dx.doi.org/10.1080/00218460903417701

The Journal of Adhesion, 86:10–24, 2010

ISSN: 0021-8464 print/1545-5823 online
DOI: 10.1080/00218460903417701

Taylor & Francis
Taylor & Francis Group

Minireview

Towards the Recombinant Production of Mussel Byssal Collagens

Anja Hagenau and Thomas Scheibel
Lehrstuhl für Biomaterialien, Universität Bayreuth,
Bayreuth, Germany

Purity and biocompatibility are prerequisites for collagen-based products in medical, pharmaceutical, and cosmetic applications. Investigation of new collagen sources and development of recombinant technologies for high-yield collagen production allow the development of novel biomaterials. Here, we highlight a fascinating natural collagenous material from marine mussels as a potential new source for biomaterial research. Further, we focus on recombinant collagen production and evaluate the feasibility to adapt these technologies for the biotechnological production of mussel byssal collagens.

Keywords: Human collagen; P4H; PreCollagens; Yeast

INTRODUCTION

Collagen is often utilized in medicinal, pharmaceutical, and cosmetic applications. The broad variety of materials morphologies (fibrils, non-woven, sponges) and its good biocompatibility enables collagen-based additives to improve the performance of medical devices like wound dressings, soft tissue implants, stents, and vascular craft coatings [1–3]. Collagen in tissue engineering can act as a tissue scaffold and has been demonstrated to enhance cell attachment, while in cosmetic applications collagen is used for dermal augmentation [4,5].

Received 20 February 2009; in final form 3 August 2009.

One of a Collection of papers honoring J. Herbert Waite, the recipient in February 2009 of *The Adhesion Society Award for Excellence in Adhesion Science, Sponsored by 3M*.

Address correspondence to Thomas Scheibel, Universität Bayreuth, Biomaterialien, FAN-D, Universitätsstr., 30, 95440 Bayreuth, Germany. E-mail: thomas.scheibel@uni-bayreuth.de

Today, most commercially available collagens are derived from vertebrates (mostly from cattle and pigs), and although they cause little immune response, there are certain concerns about its biocompatibility and homogeneity. Therefore, the recombinant production of collagens reflects an emerging field as it reflects a reproducible, safe, and animal-component free collagen source with high production yields at fairly low cost.

For improved performance, especially in medical applications, nonvertebrate collagen sources are also under investigation. One of these collagenous materials is the byssus of marine mussels which consists of collagen-like fibers embedded in a proteinaceous matrix.

Our current research focuses on investigating collagen-like proteins of mussel byssal threads and on establishing biotechnological production tools necessary for biomaterial applications. Here, we briefly review the properties of mussel byssal collagens and subsequently focus on methods of recombinant production of collagen, also feasible for mussel byssal collagen production.

Why Search for New Collagen Sources?

Collagen is the most abundant fibrous protein of the extracellular matrix of vertebrates as well as invertebrates and is the basic building block of skin, bone, and cartilage. Besides stabilizing the morphology of cells and tissue, it has a great impact on cell adhesion, growth, migration, and differentiation [6]. Due to its importance in nature, collagen from different sources has been utilized as a biomaterial for orthopedic (bone regeneration, spinal fusion), plastic (wound healing, burns), and aesthetic surgery (dermal augmentation) as well as for drug delivery, wound dressings, implant coatings, and as an additive in pharmaceuticals [1–3]. Additionally, due to its hydrating and filler abilities, collagens have been employed as an ingredient in a wide range of cosmetic products [7].

Currently, collagen is derived from bovine and porcine skin as well as from chicken waste material. However, consumers and manufactures are increasingly concerned about biological safety issues such as purity of collagen extracts. Bovine-based proteins in particular can contain biological impurities such as infectous prion proteins derived from animals with BSE (Mad Cow Disease) that encourage strict quality controls [8].

Mussel Byssi as a New Source of Collagens

Collagenous proteins from marine mussel byssal threads provide a new class of collagen material with great potential for biomaterial

research, which has been intensely studied by the group of Prof. Herbert Waite [9–11]. Mussel byssi are a unique anchorage system employed by marine mussels such as *Mytilus* (Lamarck) enabling them to settle among sea bed stones, pickets, or harbor walls. In 2005, within the European Union 361,000 tonnes of Blue Mussel were produced in aquaculture for the food industry [12], and mussel byssi are a waste product which could be recycled for collagen extraction.

The byssus consists of short, entirely extracellular proteinaceous threads which are attached to the substratum by an adhesive plaque at their distal end and connected to the mussel tissue by a stem at the proximal end (Fig. 1). Similar to tendon, 81% of the dry weight of a byssal thread are fibrilar collagenous proteins known as preCollagens (preCols) [9] and the remaining 19% can be assigned to non-collagenous matrix proteins including proximal matrix protein 1 (PTMP1) [13] and distal matrix protein 1 (DTMP1) [14] and the cuticle protein mussel foot protein-1 (mfp-1) [15]. PreCol proteins are produced in specialized collagen glands of the mussel foot and stored in secretory vesicles as short preformed fibrils arranged in smectic arrays like liquid crystals [16]. These vesicles are secreted together with the matrix and cuticle proteins into the ventral groove of the foot,

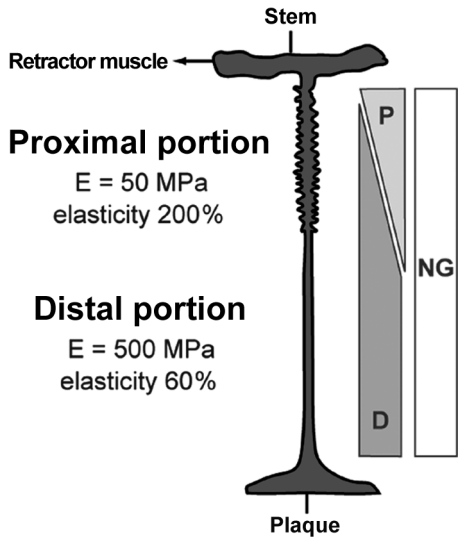

FIGURE 1 Mechanical properties of the mussel byssal thread, and the distribution of collagen-like proteins (preCols) along the thread. The basic component in the distal part is preCol D (D), while preCol P (P) is mainly found in the proximal portion, and preCol NG (NG) is homogenously distributed.

and the thread is formed by a process akin to molding. So far the exact mechanism is unclear, but the presence of post-translationally modified amino acids (Fig. 2 and see below) and the purification of the enzyme prolyl-4hydroxylase from foot tissue [17] indicates that collagen processing enzymes are involved in the fibril forming process.

Each individual byssal thread exhibits distinct mechanical properties at either end, and the transition of these properties occurs seamlessly along the thread. While the proximal part has a high elasticity (200%), the distal part is very stiff (Young's modulus = 500 MPa [9]; Fig. 1). It is supposed that the preCols are the major mediators of the distinct mechanical properties [10,18]. Protein extractions from byssal threads and analyses of cDNAs derived from mRNAs extracted from mussel feet revealed three different major preCols and some minor variants thereof (Fig. 1). The basic component of the distal part is preCol D, while preCol P is mainly found in the proximal

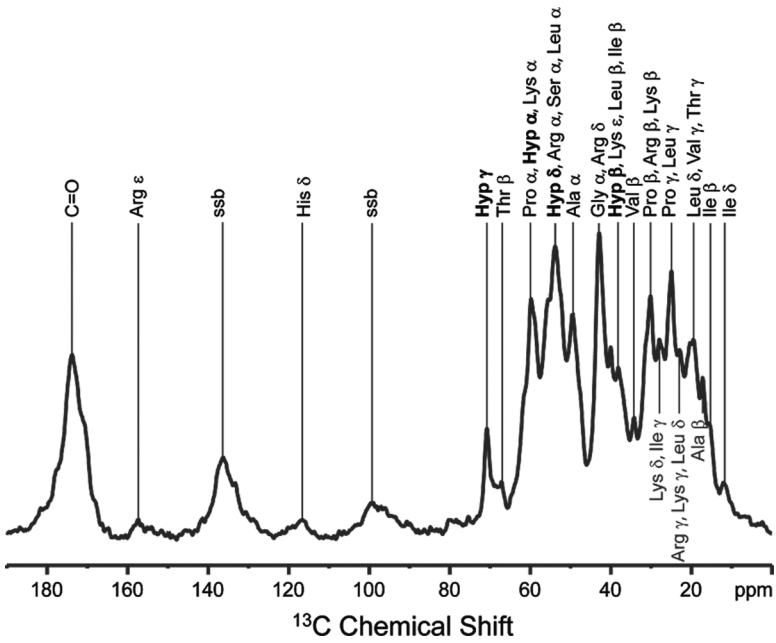

FIGURE 2 Solid state NMR spectra of entire, hydrated byssal threads. Proton-decoupled 188 MHz ^{13}C cross polarization (CP) magnetic angle spinning (MAS) NMR was measured at a MAS frequency of 7 kHz at 20°C. The signals of the major amino acids are labeled and the Hyp-signals are highlighted in bold (ssb designates spinning side bands arising on MAS). Adapted from [18]. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

portion. These two proteins are arranged in a seamless, opposed gradient along the thread reflecting the transition of mechanical properties depicted above [19–21]. In contrast, preCol NG is homogenously distributed and seems to act as a load compensating mediator between the other preCols, supporting the threads self-healing ability after stretching under tension [22,23].

Comparison of the Molecular Structure of Human and Mussel Byssal Collagen

Similar to human procollagen type I (Fig. 3a), each preCol has a modular composition. While in human procollagen type I terminal pro- and telopeptides flank the collagenous domain, mussel preCols consist of a collagen core domain, variable flanking regions, and Histidine (His)/3,4-di-hydroxyphenylalanine (DOPA)-rich regions (Fig. 3b) at each terminus [10]. Like the propeptides of procollagen type I, the His/DOPA-rich regions are responsible for the linear and lateral cross-linking of the preCols after triple helix formation by diDOPA-bonds and His-metal chelation [10,24].

It has been shown that the proteins of the distal part of the thread exhibit mostly β-structures [10,18]—a protein conformation playing

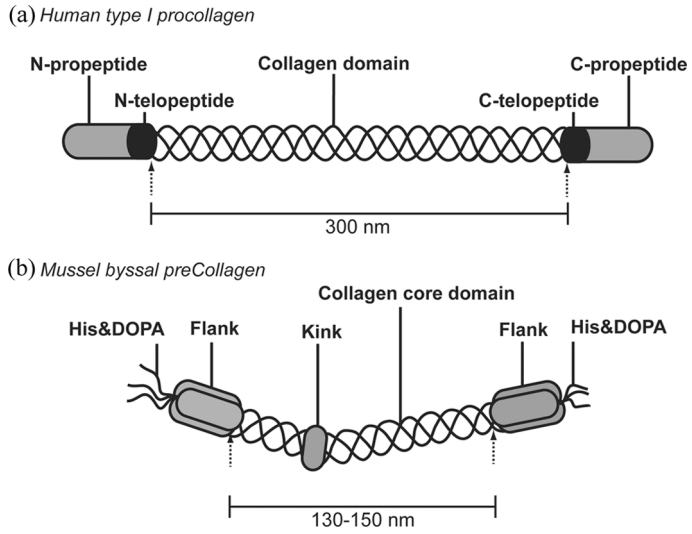

FIGURE 3 Comparison of structural composition of (a) human type I procollagen and (b) mussel byssal preCollagens. Dotted arrows indicate proteinase cleavage sides.

a major role in protein rigidity. This structure is likely based on the amino acid sequence of the flanking regions of preCol D resembling that of silk fibroin and spider dragline silk [21,25,26]. In preCol NG, also found in the distal portion of the byssal thread, plant cell wall-like motifs are present in the flanks [23] containing poly-alanine runs that are able to fold into crystalline β-sheets. In contrast, the preCol P flanks harbor motifs similar to those found in elastin and spider flagelliform silk [19,25,26] being able to form helical structures, and they are thought to be responsible for the high elasticity of the proximal thread portion.

The collagenous domain in both human procollagen type I and the preCols consists of several amino acid repeats based on the collagen typical repetitive sequence $[\text{Gly-Xaa-Yaa}]_n$ (Gly denotes glycine). While in human procollagen type I the amino acid at the Xaa-position is mostly proline (Pro), in preCols both Pro and Gly residues are found [10,20,27]. Three individual polypeptide chains fold into a helical polyproline II-like conformation resulting in a trimer with triple helical structure. Human procollagen type I consists of two α1(I)-chains and one α2(I)-chain, whereas mussel preCols are homotrimers comparable with the fibrillar human collagen type III [28]. Several Gly-deletions and distinct substitutions in the collagen motif of preCols interfere with the triple helical structure causing kinks in the molecules [10], which are not found in other fibril-forming collagens. In addition, the length of the triple helical collagen domain of preCols (130–150 nm) is only half of that of human procollagen type I (300 nm) while the diameter is 1.5 nm for both.

One important feature of collagens is the presence of post-translationally modified amino acids at the Yaa-position of the tripeptide motif. One modification is the hydroxylation of Pro to hydroxyproline (Hyp) by prolyl-hydroxylases or of lysines to hydroxylysines (Hyl) by lysyl-hydroxylases. Hydroxylysines can be additionally *O*-glycosylated with galactose and glycogalactose residues *via* the enzyme galactosyltransferase. A final processing step of collagens is the cleavage of propeptides after secretion to the intercellular space [27,29].

Although all of these processes are well studied in mammalian collagens, post-translational modifications of mussel preCols are largely unknown. The likely occurrence of post-translational hydroxylation by prolyl-hydroxylases was found by amino acid analysis [20,30] and solid state NMR studies of byssal threads (Fig. 2) [18]. Further, a protein with prolyl-hydroxylation activity has been identified (see also above) [17] and cDNAs were found in a gene library from the Mediterranean mussel *Mytilus galloprovincialis* with similarities to genes encoding the alpha and beta subunit of human prolyl4-hydroxylase (P4H) [31].

Natural Production of Collagen

Upon translation, collagen is translocated to the lumen of the endoplasmatic reticulum (ER) where it is post-translationally modified and folds into a preliminary triple helix under oxidizing conditions. Subsequently, the triple helix is transported along the secretory pathway to the extracellular space undergoing further modifications before several triple helices assemble into fibrils [28,29]. The most important modification necessary for folding into a thermally stable triple helix is hydroxylation of prolines in the Yaa-position of the collagen triplet motif by P4H within the ER. Collagen molecules without a respective proline hydroxylation mostly fold incorrectly and remain nonfunctional [32]. P4H comprises an $\alpha_2\beta_2$ tetrameric structure in vertebrates, while in *C. elegans* it consists of a $\alpha\beta$-dimer. The α-subunit of P4H contains both the substrate and co-substrate (Fe^{2+}, 2-ketoglutarate, O_2, ascorbate) binding sites as well as the catalytic site. The β-subunit is identical to the multifunctional protein disulfide isomerase (PDI) and functions as a solubilizer of the α-subunit in addition to a catalytic activity for protein folding [33,34].

Recombinant Production of Collagen

Recombinant protein production offers the possibility to generate homogeneous collagens in large quantities, avoiding animal-derived impurities obtained during extraction from cattle, pigs, or even mussel byssal threads. For decades many researchers have been working on a cost-effective and scalable technique for manufacturing recombinant human collagen with consistent quality. One major concern is the choice of a host system which produces correctly folded, stable -collagens at high yields.

Expression hosts such as bacteria and yeasts, commonly used in recombinant protein production, are *per se* not suitable for recombinant collagen, since they do not intrinsically synthesize collagen and have no P4H activity, although PDIs can be found in both systems. Other recombinant production systems employing insect cells, mammary glands, or tobacco plants also provide insufficient intrinsic hydroxylation levels.

Bacteria Hosts for Collagen Production

Prokaryotic expression hosts like *E. coli* have no ER and no hydroxylation activity. Recently, an *E. coli* strain with enhanced prolyl aminoacyl-tRNA synthase activity has been developed. Upon culturing in a hyperosmotic medium supplemented with hydroxyproline

for an increased Hyp supply, truncated human α1(I) procollagen could be produced, yielding an over-hydroxylated triple helical collagen [35]. Further, human P4H has been successfully produced in *E. coli* strains with an oxidizing cytosol [36–38]. Both studies indicate that the production of hydroxylated collagen upon co-production of active P4H might be feasible in *E. coli*.

However, bacterial production systems like *E. coli* show several limitations. Human collagen and even the preCols are large molecules often exceeding the capability of bacterial host systems. Additionally, these proteins comprise repetitive sequences which might undergo recombination events through the bacterial transcription-translation machinery resulting in truncated gene products. Based on results with repetitive spider silk proteins [26,39,40], genetically designed collagen fragments adapted to the bacterial codon usage could be produced in *E. coli* strains with a knock out of the *rec*A gene preventing homologous recombination (Fig. 4).

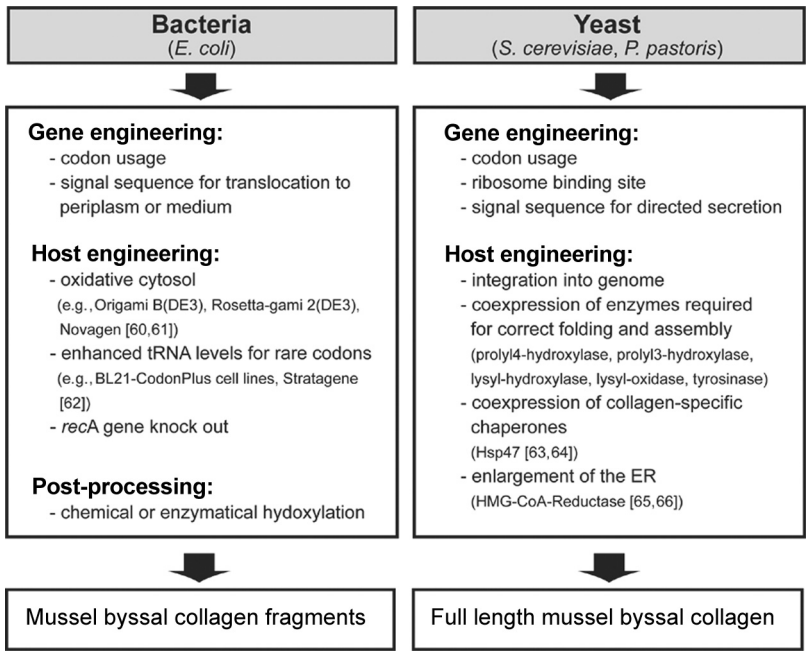

FIGURE 4 Strategies of engineering of bacterial and yeast production systems for recombinant mussel byssal collagens.

Yeast Hosts for Collagen Production

Yeasts are among the best-characterized model organisms in microbiology, and have been established as hosts for biotechnological production of a variety of heterologous proteins [41–43].

Previously, the baker's yeast, *Saccharomyces cerevisiae*, has been employed for recombinant collagen production. Human α1(I) and α2(I) procollagen can be co-produced with the α- and β-subunits of P4H derived from various different species. The highest yield of natively folded collagen fibrils has been obtained by co-expressing plasmids encoding α1(I)/α2(I) procollagen and chicken P4H [44]. The amount of produced recombinant collagen could be further increased by integrating the P4H genes into the genome of the host (Table 1). In other studies, production of truncated recombinant human α1(I) procollagen consisting of the core domain and terminal telopeptides, achieved highest expression levels by fermentation, and the produced type I heterotrimers were able to form collagen fibrils [45].

However, the practical use of the *S. cerevisiae* expression system is limited, especially since recombinant proteins produced therein are typically hyperglycosylated: *N*-linked carbohydrates terminated by mannose are connected to the peptide chain, which may be allergenic. Additionally, instabilities of recombinant strains render the production process less reliable due to the use of episomal vectors [42].

Alternatively, the methylotrophic yeast *Pichia pastoris* has been established for industrial high level production of collagen, since it can be cultured at extremely high cell densities (500 OD_{600} U mL^{-1}). *P. pastoris* is further able to *O*- and *N*-glycosylate-appropriate hydroxyl groups [41]. Analysis of *P. pastoris* producing P4H α- and β-subunits and procollagen type III revealed that synthesis of stable and active tetrameric P4H requires co-production of collagen, and thermally stable, well-assembled collagen needs co-production of α- and β-subunits of P4H [46,47]. Additionally, employing the pre-prosequence of α-mating factor (αMF) of *S. cerevisiae* instead of the original human signal sequence in the β-subunit of P4H improved its translocation into the ER, followed by increased enzyme activity and yield of collagen production [46] with correctly folded collagen trimers and Hyp-contents identical to nature (Table 1) [48–50].

During collagen production in *P. pastoris*, single chains of truncated (45 kDa) human α1(I) collagen have been efficiently secreted into the medium, whilst 90 kDa fragments and properly folded triple helical molecules were retained in the ER lumen [51,52]. In human fibroblasts, triple helical collagen is transported from the ER to the Golgi compartment by special tubular-saccular structures with a size larger

TABLE 1 Comparision of Expression Systems for the Recombinant Production of Collagen

Host	Collagen type	P4H coexpression	Yield	Ratio Hyp/Pro	Result	Ref.
Bacteria						
Escherichia coli	h α1(I) (truncated)	None, hyperosmotic media with Hyp	n/a	1.0	Homotrimer	[31]
Yeast						
Saccharomyces cerevisiae	h pro α1(I) + pro α2(I)	ch αP4H + βP4H	4 μg/mg total protein	0.39	Type I heterotrimer	[39]
	h α1(I) + α2(I)	ch αP4H + βP4H	29.8 μg/mg total protein (fermentation)	0.23	Type I heterotrimer	[40]
Pichia pastoris	h pro α1(III)	h αP4H + βP4H (+αMF)	15 mg/l	0.44	Type III homotrimer, disulphide linked	[41]
	h pro α1(I) + pro α2(I)	h αP4H + βP4H (+αMF)	0.2–0.6 g/l (fermentation)	Identical to non-recombinant collagens	Type I heterotrimer Type II homotrimer Type III homotrimer	[43]
	h pro α1(II) h pro α1(III)					
	h pro α1(I) + pro α2(I)	h αP4H + βP4H	1.1 g/l	0.47	Type I heterotrimer	[45]
	h pro α1(II)		0.7 g/l	n/a	Type II homotrimer	
	h pro α1(III)		1.5 g/l	0.56	Type III homotrimer	
Eukaryotic cells						
Spodoptera frugiperda (Sf9, Sf21)	h pro α1(III)	h αP4H + βP4H	40 mg/l cell suspension	0.5	Type III homotrimer	[48]
	h pro α1(I)	h αP4H + βP4H	60 mg/l cell suspension	n/a	Type I homotrimer	[49]
Trichoplusia ni (HighFive)	h pro α1(I) + pro α2(I)	h αP4H + βP4H	40 mg/l cell suspension	n/a	Type I heterotrimer	[49]

(*Continued*)

TABLE 1 Continued

Host	Collagen type	P4H coexpression	Yield	Ratio Hyp/Pro	Result	Ref.
Transgenetic organisms						
Mus musculus (mouse)	α2(I) (truncated)	h αP4H + βP4H	50–200 mg/l	n/a	Homotrimer,	[50]
	h pro α1(I)	None	1–8 g/l (not purified)	0.17	Type I homotrimer	[51]
Bombyx mori (silkworm)	h pro α1(III) minichain	None	2.52 mg/cocoon 36.7 μg/mg total protein	low	Type III homotrimer	[52]
Nicotinia spec. (tobacco)	h pro α1(I)	None	3 mg/100 g leafs	unhydroxylated	Type I homotrimer, disulphide-bonded	[53]
	h pro α1(I)	*Ce* αP4H +m βP4H	2 mg/100 g leafs	0.36	Type I homotrimer	[54]

Abbreviations: h-human, ch-chicken, m-mouse, *Ce-Caenorhabditis elegans*, +αMF- with pre-prosequence of the α mating factor.

than 300 nm. By contrast, COP II-coated *cis*-Golgi vesicles in *P. pastoris* are too small (60–90 nm) to encapsulate properly folded recombinant collagens.

Eukaryotic Cells for Collagen Production

Eukaryotic cell lines allowing high yields of recombinant protein production are, for example, baculovirus infected insect cells from the fall armyworm *Spodoptera frugiperda* (Sf9, Sf21) or *Trichoplusia ni* (HighFive). This expression system has been used to produce natively folded, disulfide-bonded, and correctly modified collagens (Table 1) [53,54]. Although insect cells reveal an intrinsic P4H activity, additional transfection with viruses encoding P4H was necessary to increase both the yield and stability of the collagen triple helix. The baculovirus system provides the production of sufficient amounts of native collagens and preCols for basic protein characterization, but due to apparently low productivity and high costs it is not ideal for commercial production.

Transgenic Organisms for Collagen Production

Mammary glands of transgenic animals provide a suitable expression system for collagens. Several human proteins have been produced in a biologically active form in the milk of sheep, mice, rabbits, and pigs. A truncated α2(I) collagen was co-produced with human P4H in mammary glands of mice (*Mus musculus*) (Table 1) [55]. A relatively high amount (1–8 mg/mL) of incompletely hydroxylated full length human α1(I) procollagen, able to form homotrimers, was obtained in mouse milk [56]. Recombinant production of human α1(III) procollagen in silkworm *Bombyx mori* silk glands resulted in incorporation of collagens into the silk cocoons, but the collagens had low levels of hydroxyproline [57]. In transgenic tobacco plants unhydroxylated type I homotrimer has been produced [58], and the Hyp-content could be increased by co-production of the α-subunit of P4H from *C. elegans* together with the mouse P4H β-subunit [59]. Due to long generation times and inadequate Hyp-content, transgenic organisms have so far not been used for large scale recombinant production of collagens.

Outlook: Recombinant Byssal Collagens as New Biomaterials

Among biopolymers, preCols from mussel byssal threads provide a new functional class. They comprise collagenous characteristics as well as sophisticated mechanical properties. PreCols might offer the

possibility to create novel biomaterials for a wide range of medical and other applications.

The individual proteins preCol D and NG and processed materials thereof could increase the rigidity of engineered bone scaffolds, while preCol P might provide elasticity for ligament implants. Additionally, preCol-derived surface coatings of implants might increase their mechanical stability and cell compatibility. Similar to human collagen, preCols could be recombinantly produced with a consistent quality in large quantities and high purity.

Due to long generation times and high costs, transgenic animals are not attractive for large scale production of recombinant mussel byssal collagens. Based on our experiences on recombinant production of highly repetitive structural proteins such as spider silks, we suppose that the most promising host systems for the production of proteins for basic investigations are insect cells, whereas *P. pastoris* and *E.coli* might be suitable for large scale production. Strategies for engineering highly productive host systems are summarized in Fig. 4.

Despite the similarities to human collagen, preCols additionally contain protein domains (elastin-, silk-like flanks, as well as His/DOPA regions) which strongly influence the protein properties such as solubility and aggregation behaviour. We assume these additional domains might cause unexpected problems during production, purification, and subsequent processing into functional biomaterials. In our opinion, the production of mussel byssal collagens remains a biotechnological challenge and we believe that, once this has been tackled, recombinant collagens derived from mussel byssal preCols might advance to a biopolymer for interesting applications.

ACKNOWLEDGMENTS

This work was supported by the Graduate Programme "Material Science of Complex Interfaces" of the "Elitenetzwerk Bayern" (AH) and the DFG (SCHE 603/7-1)(TS). Special thanks to Prof. Daniel Huster for NMR data and for proof reading to J. Hardy and A. Golser and the members of the Fiberlab for discussions and critical comments.

REFERENCES

[1] Chevallay, B. and Herbage, D., *Med. Biol. Eng. Comput.* **38**, 211–218 (2000).
[2] Lee, C. H., Singla, A., and Lee, Y., *Int. J. Pharm.* **221**, 1–22 (2001).
[3] Purna, S. K. and Babu, M., *Burns* **26**, 54–62 (2000).
[4] Baumann, L., Kaufman, J., and Saghari, S., *Dermatol. Ther.* **19**, 134–140 (2006).
[5] Sadick, N. and Sorhaindo, L., *Expert Rev. Med. Devices* **4**, 559–565 (2007).

[6] Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P., *Molekularbiologie der Zelle*, (Wiley-VCH Verlag GmbH, Weinheim, 2004), 4th ed.
[7] GdCH Lebensmittelchemische Gesellschaft, AG Kosmetische Mittel, *Datenblätter zur Bewertung der Wirksamkeit von Wirkstoffen in kosmetischen Mitteln*, (Freiburg, 2006), Ed. 2006, http://www.gdch.de/strukturen/fg/lm/ag/kosmetik.htm
[8] O'Grady, J. E. and Bordon, D. M., *Adv. Drug Deliv. Rev.* **55**, 1699–1721 (2003).
[9] Waite, J. H., Vaccaro, E., Sun, C., and Lucas, J. M., *Philos. Trans. R Soc. Lond. B Biol. Sci.* **357**, 143–153 (2002).
[10] Waite, J. H., Qin, X. X., and Coyne, K. J., *Matrix Biol.* **17**, 93–106 (1998).
[11] Harrington, M. J. and Waite, H. J., *Fibrous Proteins*, (Landes Bioscience, Austin, 2008), Ch. 3, pp. 30–45.
[12] European Commission, Facts and figures on the CFP – Basic data on the common fisheries policy, (Office for Official Publications of the European Communities, Luxembourg, 2008), Ed. 2008, pp. 18.
[13] Sun, C., Lucas, J. M., and Waite, J. H., *Biomacromolecules* **3**, 1240–1248 (2002).
[14] Waite, J. H., Lichtenegger, H. C., Stucky, G. D., and Hansma, P., *Biochemistry* **43**, 7653–7662 (2004).
[15] Sun, C. and Waite, J. H., *J. Biol. Chem.* **280**, 39332–39336 (2005).
[16] Zuccarello, L. V., *J. Ultrastruct. Res.* **73**, 135–147 (1980).
[17] Marumo, K. and Waite, J. H., *J. Exp. Zool.* **244**, 365–374 (1987).
[18] Hagenau, A., Scheidt, H. A., Serpell, L., Huster, D., and Scheibel, T., *Macromol. Biosci.* **9**, 162–168 (2009).
[19] Coyne, K. J., Qin, X. X., and Waite, J. H., *Science* **277**, 1830–1832 (1997).
[20] Qin, X. and Waite, J. H., *J. Exp. Biol.* **198**, 633–644 (1995).
[21] Qin, X. X., Coyne, K. J., and Waite, J. H., *J. Biol. Chem.* **272**, 32623–32627 (1997).
[22] Harrington, M. J. and Waite, J. H., *J. Exp. Biol.* **210**, 4307–4318 (2007).
[23] Qin, X. X. and Waite, J. H., *Proc. Natl. Acad. Sci. USA* **95**, 10517–10522 (1998).
[24] Vaccaro, E. and Waite, J. H., *Biomacromolecules* **2**, 906–911 (2001).
[25] Hardy, J. G., Roemer, L. M., and Scheibel, T. R., *Polymer* **49**, 4309 (2008).
[26] Scheibel, T., *Microb. Cell Fact* **3**, 14 (2004).
[27] Voet, D. J., Voet, J. G., and Pratt, C. W., *Lehrbuch der Biochemie*, (Wiley-VCH Verlag GmbH, Weinheim, 2002), 1st ed.
[28] Kadler, K. E., Holmes, D. F., Trotter, J. A., and Chapman, J. A., *Biochem. J.* **316** (Pt 1), 1–11 (1996).
[29] Canty, E. G. and Kadler, K. E., *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **133**, 979–985 (2002).
[30] Benedict, C. V. and Waite, J. H., *J. Morphol.* **189**, 261–270 (1986).
[31] MytiBase, http://mussel.cribi.unipd.it/.
[32] Berg, R. A. and Prockop, D. J., *Biochem. Biophys. Res. Commun.* **52**, 115–120 (1973).
[33] Kivirikko, K. I. and Myllyharju, J., *Matrix Biol.* **16**, 357–368 (1998).
[34] Wilson, R., Lees, J. F., and Bulleid, N. J., *J. Biol. Chem.* **273**, 9637–9643 (1998).
[35] Buechter, D. D., Paolella, D. N., Leslie, B. S., Brown, M. S., Mehos, K. A., and Gruskin, E. A., *J. Biol. Chem.* **278**, 645–650 (2003).
[36] Kersteen, E. A., Higgin, J. J., and Raines, R. T., *Protein Expr. Purif.* **38**, 279–291 (2004).
[37] Neubauer, A., Neubauer, P., and Myllyharju, J., *Matrix Biol.* **2 4**, 59–68 (2005).
[38] Neubauer, A., Soini, J., Bollok, M., Zenker, M., Sandqvist, J., Myllyharju, J., and Neubauer, P., *J. Biotechnol.* **128**, 308–321 (2007).
[39] Huemmerich, D., Helsen, C. W., Quedzuweit, S., Oschmann, J., Rudolph, R., and Scheibel, T., *Biochemistry* **43**, 13604–13612 (2004).
[40] Vendrely, C. and Scheibel, T., *Macromol. Biosci.* **7**, 401–409 (2007).
[41] Cereghino, J. L. and Cregg, J. M., *FEMS Microbiol. Rev.* **24**, 45–66 (2000).

[42] Gellissen, G., Melber, K., Janowicz, Z. A., Dahlems, U. M., Weydemann, U., Piontek, M., Strasser, A. W., and Hollenberg, C. P., *Antonie Van Leeuwenhoek* **62**, 79–93 (1992).
[43] Sudbery, P. E., *Curr. Opin. Biotechnol.* **7**, 517–524 (1996).
[44] Toman, P. D., Chisholm, G., McMullin, H., Giere, L. M., Olsen, D. R., Kovach, R. J., Leigh, S. D., Fong, B. E., Chang, R., Daniels, G. A., Berg, R. A., and Hitzeman, R. A., *J. Biol. Chem.* **275**, 23303–23309 (2000).
[45] Olsen, D. R., Leigh, S. D., Chang, R., McMullin, H., Ong, W., Tai, E., Chisholm, G., Birk, D. E., Berg, R. A., Hitzeman, R. A., and Toman, P. D., *J. Biol. Chem.* **276**, 24038–24043 (2001).
[46] Vuorela, A., Myllyharju, J., Nissi, R., Pihlajaniemi, T., and Kivirikko, K. I., *EMBO J.* **16**, 6702–6712 (1997).
[47] Vuorela, A., Myllyharju, J., Pihlajaniemi, T., and Kivirikko, K. I., *Matrix Biol.* **18**, 519–522 (1999).
[48] Myllyharju, J., Nokelainen, M., Vuorela, A., and Kivirikko, K. I., *Biochem. Soc. Trans.* **28**, 353–357 (2000).
[49] Nokelainen, M., Tu, H., Vuorela, A., Notbohm, H., Kivirikko, K. I., and Myllyharju, J., *Yeast* **18**, 797–806 (2001).
[50] Olsen, D., Yang, C., Bodo, M., Chang, R., Leigh, S., Baez, J., Carmichael, D., Perala, M., Hamalainen, E. R., Jarvinen, M., and Polarek, J., *Adv. Drug Deliv. Rev.* **55**, 1547–1567 (2003).
[51] Keizer-Gunnink, I., Vuorela, A., Myllyharju, J., Pihlajaniemi, T., Kivirikko, K. I., and Veenhuis, M., *Matrix Biol.* **19**, 29–36 (2000).
[52] Pakkanen, O., Pirskanen, A., and Myllyharju, J., *J. Biotechnol.* **123**, 248–256 (2006).
[53] Lamberg, A., Helaakoski, T., Myllyharju, J., Peltonen, S., Notbohm, H., Pihlajaniemi, T., and Kivirikko, K. I., *J. Biol. Chem.* **271**, 11988–11995 (1996).
[54] Myllyharju, J., Lamberg, A., Notbohm, H., Fietzek, P. P., Pihlajaniemi, T., and Kivirikko, K. I., *J. Biol. Chem.* **272**, 21824–21830 (1997).
[55] John, D. C., Watson, R., Kind, A. J., Scott, A. R., Kadler, K. E., and Bulleid, N. J., *Nat. Biotechnol.* **17**, 385–389 (1999).
[56] Toman, P. D., Pieper, F., Sakai, N., Karatzas, C., Platenburg, E., de Wit, I., Samuel, C., Dekker, A., Daniels, G. A., Berg, R. A., and Platenburg, G. J., *Transgenic Res.* **8**, 415–427 (1999).
[57] Tomita, M., Munetsuna, H., Sato, T., Adachi, T., Hino, R., Hayashi, M., Shimizu, K., Nakamura, N., Tamura, T., and Yoshizato, K., *Nat. Biotechnol.* **21**, 52–56 (2003).
[58] Ruggiero, F., Exposito, J. Y., Bournat, P., Gruber, V., Perret, S., Comte, J., Olagnier, B., Garrone, R., and Theisen, M., *FEBS Lett.* **469**, 132–136 (2000).
[59] Merle, C., Perret, S., Lacour, T., Jonval, V., Hudaverdian, S., Garrone, R., Ruggiero, F., and Theisen, M., *FEBS Lett.* **515**, 114–118 (2002).
[60] Zhou, X., Tan, F. K., Milewicz, D. M., Guo, X., Bona, C. A., and Arnett, F. C., *J. Immunol.* **175**, 4555–4560 (2005).
[61] Park, M. O., Mizutani, T., and Jones, P. R., *J. Bacteriol.* **189**, 7281–7289 (2007).
[62] Pugh, R. A., Honda, M., Leesley, H., Thomas, A., Lin, Y., Nilges, M. J., Cann, I. K., and Spies, M., *J. Biol. Chem.* **283**, 1732–1743 (2008).
[63] Koide, T., Nishikawa, Y., Asada, S., Yamazaki, C. M., Takahara, Y., Homma, D. L., Otaka, A., Ohtani, K., Wakamiya, N., Nagata, K., and Kitagawa, K., *J. Biol. Chem.* **281**, 11177–11185 (2006).
[64] Koide, T., Asada, S., Takahara, Y., Nishikawa, Y., Nagata, K., and Kitagawa, K., *J. Biol. Chem.* **281**, 3432–3438 (2006).
[65] Koning, A. J., Roberts, C. J., and Wright, R. L., *Mol. Biol. Cell.* **7**, 769–789 (1996).
[66] Wright, R., Basson, M., D'Ari, L., and Rine, J., *J. Cell. Biol.* **107**, 101–114 (1988).