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Triple helix assembly and processing of human collagen produced in transgenic tobacco plants

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Abstract The use of tobacco plants as a novel expression system for the production of human homotrimeric collagen I is presented in this report. Constructs were engineered from cDNA encoding the human proα1(I) chain to generate transgenic tobacco plants expressing collagen I. The recombinant proα1(I) chains were expressed as disulfide-bonded trimers and were shown to fold into a stable homotrimeric triple helix. Moreover, the recombinant procollagen was subsequently processed to collagen as it occurs in animals. Large amounts of recombinant collagen were purified from field grown plant material. The data suggest that plants are a valuable alternative for the recombinant production of collagen for various medical and scientific purposes.

Key words: Recombinant collagen; Transgenic plant; Triple helix assembly

1. Introduction

Collagens form a family of extracellular matrix proteins found in all connective tissues. They represent 30% of total body proteins in mammalians, the most abundant components being the fibrillar collagens I–III which make up the cross-striated fibril network of extracellular matrices. Collagen molecules comprise three polypeptides, referred to as α chains, which assemble to form triple helical domains. The α chains contain the repeating triple sequence Gly-X-Y in which X and Y are frequently proline and hydroxyproline residues, respectively. This sequence is required for the correct formation of the triple helix since only the glycyl residue can fit at the center of the helix and the imino acid residues are essential for triple helix folding and stabilization [1].

Fibrillar collagen I is the most widely distributed in tissues, where it occurs with the chain composition $[\alpha1(I)]_2\alpha2(I)$. However, in addition to this form, tissues also contain substantial amounts of the homotrimer $[\alpha1(I)]_3$. Both molecules have been shown to form a stable triple helix. The molecule is synthesized as a procollagen, triple helix formation being initiated by C-propeptide interactions. Cleavage of the N- and C-terminal propeptides from the secreted collagen molecule by specialized enzymes is a prerequisite for fibril assembly [2].

Collagen I is primarily a structural protein but it is also involved in a number of biological events such as early development, organogenesis, cell attachment and proliferation, hemostasis, wound healing and tissue remodelling. The mechanical properties of this protein, its biological characteristics and its poor immunogenicity render it suitable for natural-based biomaterial use [3]. Indeed, collagen I is widely exploited for medical use, cosmetics, therapeutics and also as gelatin (denatured collagen) in food. However, this material is mainly extracted from animal tissues, which entails potential viral or infectious agent contamination risks and allergic reaction problems. Because the development of an efficient expression system for such proteins would have numerous applications, different cell systems have been developed to produce well-folded recombinant collagens. Indeed, fibrillar collagens have already been expressed in mammalian cells [4–6], insect cells [7–9] and in yeast [10]. Recently, the production in mouse milk of a chimeric mini-procollagen molecule has also been described [11]. The use of plants as an expression system represents a breakthrough for the production of mammalian proteins. Amongst others, complex functional proteins such as immunoglobulins [12] and hemoglobin [13] have been successfully produced in transgenic plants. Here, we show that tobacco plants, transformed by cDNA encoding the human preproα1 chain of collagen I, are able to produce fully processed triple helical molecules and thus may represent a suitable alternative source of collagen for cost-effective large-scale production.

2. Materials and methods

2.1. Constructions and transformation of tobacco plants

The cDNA clones 1α3 and α22, together encoding the complete human proα1(I) collagen chain, were obtained by screening a MG63 (human osteosarcoma cell line, ATCC, 1427) cDNA library [14] with two γ-^{32}P-labelled oligonucleotides (nucleotides 152–171 and 4268–4274 of the human proα1(I) transcript, numbering from the translation initiation site). To obtain the three constructs used in this study, the 1554 bp *Bam*HI-*Eco*RI fragment from the cDNA clone α22 was cloned into pBluescriptSK (Stratagene) (clone 1). The *Eco*RI-*Hin*dIII PCR fragment encoding the carboxy-terminus of the proα1(I) chain was ligated into the *Eco*RI and *Hin*dIII sites of clone 1 in order to obtain clone 2. Clone 3 was generated by cloning a 1441 bp *Xba*I-*Bam*HI PCR fragment into the plasmid pUC19. This PCR fragment contains the coding sequence for the amino-telopeptide and the amino-terminus of the central triple helix of the proα1(I) chain. The *Dra*III-*Bam*HI fragment was ligated to the *Dra*III-*Bam*HI sites of clone 3 to obtain clone 4. Clone 5 was generated by ligating the *Xba*I-*Bam*HI fragment of clone 4 to the same sites in clone 2. A 473 bp *Sac*II-*Nhe*I PCR fragment encoding the signal peptide and the N-propeptide of the preproα1(I) chain was ligated to the same sites in clone 5 and the resultant construct, α1(I), encodes the complete preproα1(I) chain. The *Nhe*I PCR-generated restriction site sub-

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stitutes the Asn-158 and Phe-159 to Lys and Leu residues, respectively (the residues are numbered from the first methionine). A *Sac*II-*Nhe*I PCR fragment encoding the plant signal peptide of PR-protein (pathogenesis-related protein), referred to as PRS, [15] was ligated to the *Sac*II-*Nhe*I sites of clone 5 to obtain the PRS(ΔN-pro) construct. It encodes the signal peptide PRS and the complete proα1(I) chain except the N-propeptide sequence. For the last construct, a 407 bp *Nhe*I-*Nhe*I PCR fragment encoding the N-propeptide of the proα1(I) chain was ligated to the *Nhe*I site of the construct PRS(ΔN-pro). The resultant construct PRSα1(I) encodes the complete proα1(I) chain in which the natural signal peptide is replaced by PRS. The different constructs are illustrated in Fig. 1. All the PCR fragments have been sequenced.

Constructs were digested by *Hin*dIII and ligated to the binary plasmid pBIOC21 [16]. This plasmid contains the recombinant coding region under the control of the enhanced 35S promoter and the 35S terminator of the cauliflower mosaic virus. The selectable marker gene NPT2 is under the control of promoter NOS and the NOS terminator of *Agrobacterium tumefaciens* [17]. The resultant constructs were termed pBIOC-α1(I), pBIOC-PRS(ΔN-pro) and pBIOC-PRSα1(I), respectively. Transformations were achieved by infecting leaf discs of tobacco with recombinant *A. tumefaciens* as previously described [17]. Kanamycin-resistant transformants were selected and regenerated in order to screen plantlets expressing collagen I.

2.2. Extraction and purification of collagens

2.2.1. Collagen I standards. Collagens were extracted from fetal calf bones by pepsin digestion and separated by salt fractionation as previously described [18]. The 0.7 M NaCl and 0.9 M NaCl precipitates were shown to contain the heterotrimeric form $[\alpha1(I)]_2\alpha2(I)$ and the $[\alpha1(I)]_3$ homotrimer, respectively (data not shown).

2.2.2. Recombinant collagen. Plantlets (leaves and stems) were frozen in liquid nitrogen and homogenized in a freezer mill (Spex Industries, Metuchen, NJ, USA). Total protein was extracted in 50 mM Tris (pH 7.4), 150 mM NaCl, 2.5 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 1 mM *N*-ethylmaleimide at 4°C overnight and centrifuged to eliminate insoluble materials. Supernatants were analyzed by immunoblotting as described below.

Frozen leaves and stems of transformed mature plants were powdered under liquid nitrogen as described above and the homogenate was extracted in 0.5 M acetic acid containing 0.2 M NaCl for 60 h at 4°C. After removing insoluble material by centrifugation, the supernatant containing the recombinant collagen was salt-fractionated at 0.4 M and 0.9 M NaCl. The 0.9 M NaCl precipitate, containing the recombinant collagen, was dissolved in and dialyzed against 0.1 M acetic acid and stored at −20°C until used.

2.3. Western blot analysis

Samples were analyzed by 6% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing or non-reducing conditions (when indicated) followed by electrotransfer onto polyvinylidene difluoride membranes (Immobilon-P, Millipore) overnight in 10 mM CAPS (pH 11), 5% methanol. After saturation, membranes were incubated with polyclonal antibodies against human collagen I (kindly provided by Dr. D Hartman, Institut Pasteur, Lyon, France) followed by secondary antibodies conjugated to alkaline phosphatase. Immunoblots were revealed with the alkaline phosphatase conjugate substrate kit from Bio-Rad.

2.4. Analytical and electron microscopy methods

To determine N-terminal amino acid sequences, samples were subjected to SDS–PAGE electrophoresis and then electrotransfered. The bands of interest were excised from the membrane and amino acid sequencing was performed by automated Edman degradation using an Applied Biosystems 473A protein sequencer.

For amino acid composition analysis, the 0.9 M NaCl precipitate containing the recombinant collagen dissolved in 0.1 M acetic acid was heated at 60°C for 15 min prior to further purification by reverse phase HPLC on a aquapore C8 RP300 column using an aqueous acetonitrile gradient in the presence of 0.1% trifluoroacetic acid. The purified recombinant α1(I) chains appeared as a single peak and exhibited the expected molecular mass as analyzed by 6% SDS–PAGE (not shown). The same purification procedure was carried out on bovine collagen I homotrimer samples. The amino acid composition, performed on both samples, was determined after hydrolysis under vacuum (6 N HCl, 115°C, 24 h) in presence of 2-mercaptoethanol in a Pico Tag system (Waters) with a Beckman amino acid analyzer.

For rotary shadowing, samples were diluted to 10 μg/ml with 0.1 M acetic acid, mixed with glycerol (1:1), sprayed onto freshly cleaved mica sheets and immediately placed on the holder of a MED 010 evaporator (Balzers). Rotary shadowing was carried out as previously described [19]. Observations of replicas were performed with a Philips CM120 microscope at the 'Centre de Microscopie Electronique Appliquée à la Biologie et à la Géologie, Université Claude Bernard, Lyon I'.

2.5. Circular dichroism

Triple helical conformation and thermal stability of the recombinant homotrimer were analyzed by circular dichroism. Spectra were recorded at 10°C in 100 mM acetic acid on a CD6 Jobin Yvon spectropolarimeter equipped with a variable temperature unit. Samples of recombinant collagen and, for comparison, bovine homotrimer were dissolved in 100 mM acetic acid. Heat denaturation, when necessary, was performed immediately before the analysis. Measurements were performed with a 0.1 cm path length cell at a constant rate of 10 nm/min with a 0.2 nm resolution. Thermal transition curves were measured on both samples at a concentration of 185 μg/ml by monitoring [θ] 225 nm as a function of temperature at a heating rate of 10°C/h.

3. Results and discussion

3.1. Analysis of recombinant collagen I expressed in plantlets

Full-length cDNA encoding the human proα1(I) chain was used to engineer three different constructs and to generate transgenic tobacco plants expressing collagen I (Fig. 1).

Over 20 independent primary transformants were obtained for each construct. Plantlets were screened for the expression of recombinant collagen I by Western blotting using polyclonal antibodies against human collagen I. Two bands were detected in protein extracts transformed with either pBIOC-α1(I) (not shown) or pBIOC-PRSα1(I) (Fig. 2A, lane 1). These two recombinant α1(I)-derived forms migrate at about 160 kDa and 140 kDa, respectively. With the construct lacking the N-propeptide, pBIOC-PRS(ΔN-pro), two polypeptides were also detected but the electrophoretic pattern is different. The slower band migrated at 140 kDa and the faster one at

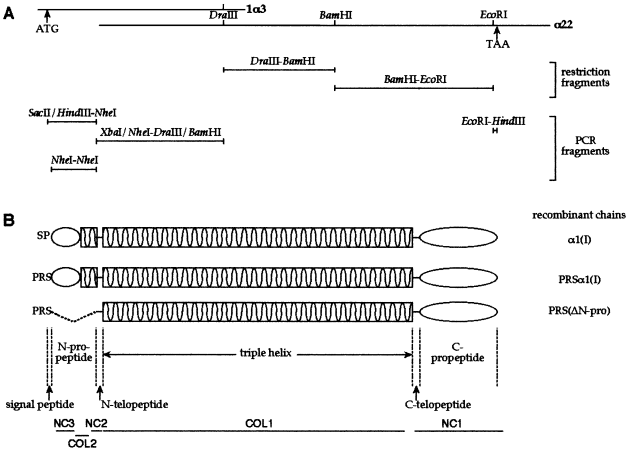

Fig. 1. Human proα1(I) constructs. (A) Restriction fragments and PCR products used for the generation of the proα1(I) expression constructs are represented below the overlapping cDNA clones 1α3 and α22. (B) depicts the composition of the different engineered protein constructs. SP, signal peptide; PRS, plant signal peptide; NC1, NC2 and NC3, non-collagenous domains; COL1 and COL2, collagenous domains. Domains are numbered from the carboxy-terminus.

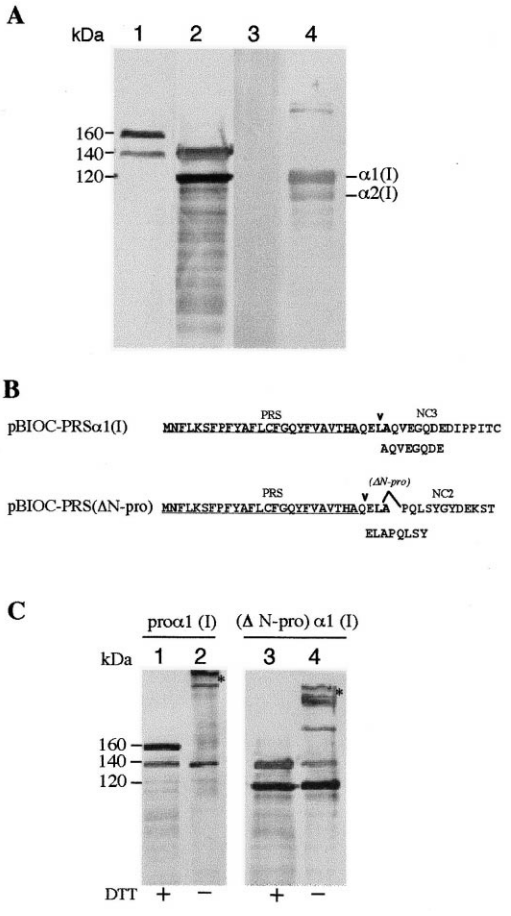

Fig. 2. A: Western blot of recombinant α1(I) collagen in crude extracts from tobacco plantlets. Lane 1: transgenic pBIOC-PRSα1(I). Lane 2: transgenic pBIOC-PRS(ΔN-pro). Lane 3: wild-type plant. Lane 4: purified bovine $[\alpha1(I)]_2\alpha2(I)$ collagen I. B: N-terminal peptide sequences of recombinant proα1(I) chain-derived forms obtained from pBIOC-PRSα1(I) and pBIOC-PRS(ΔN-pro) transgenic tobacco protein extracts. Residues derived from N-terminal sequencing are aligned with the sequence deduced from the cDNA of each construct. The modifications made for engineering the constructs are bold face type and the signal peptide sequence is underlined. Arrowheads indicate the PRS cleavage site determined by N-terminal sequencing. C: Formation of inter-chain disulfide bonds analyzed by Western blotting of the recombinant proα1(I)-derived forms under reducing (lanes 1 and 3) and non-reducing conditions (lanes 2 and 4). Asterisks indicate the running positions of trimers. Molecular masses (in kDa) are indicated for recombinant protein forms on the left of A and C.

120 kDa (Fig. 2A, lane 2). The 120 kDa band nearly co-migrated with the fully processed bovine α1(I) chain and thus the 160 kDa and 140 kDa bands probably correspond to intermediate processed forms. N-terminal sequence analysis of the 140 kDa (Fig. 2A, lane 1) and the 120 kDa (Fig. 2A, lane 2) bands obtained with the pBIOC-PRSα1(I) and pBIOC-PRS(ΔN-pro) constructs, respectively, indicated that they both start with the first amino acid after the plant signal peptide sequence (Fig. 2B) and thus show that these two bands were not processed at their N-terminal extensions. Under non-reducing conditions, only the upper bands obtained with each construct migrated as high molecular mass products corresponding to trimers (Fig. 2C). These results indicated that whatever the construct was, the recombinant collagen was synthesized in plantlets as a precursor which was disulfide-linked through its C-propeptides. This precursor underwent C-terminal processing that removed the C-propeptides but retained, when present in the construct, the complete N-propeptide.

3.2. *Purification of recombinant collagen I from mature transgenic plants*

The amount of expressed collagen I was shown to vary from one plant to another. Therefore, only selected plants (transformed with pBIOC-PRS(ΔN-pro) or pBIOC-PRSα1(I)) for which the collagen expression level was estimated to be more than 10 mg/100 g of powdered plants were grown to maturity for carrying out purification of high quantities of recombinant protein. The recombinant collagen was thus extracted and purified from field grown plants mature plant extracts on the basis of the known physical and chemical properties of collagens. Collagen is acid soluble while acidification causes most of the plant proteins to precipitate. Raising the salt concentration allowed further separation of recombinant collagen from the remaining proteins as judged by 5–15% SDS–PAGE (Fig. 3A). Purity of recombinant collagen I in the 0.9 M NaCl precipitate is estimated to be about 90% based on the percentage of glycine and proline residues obtained with amino acid composition analysis. Approxi-

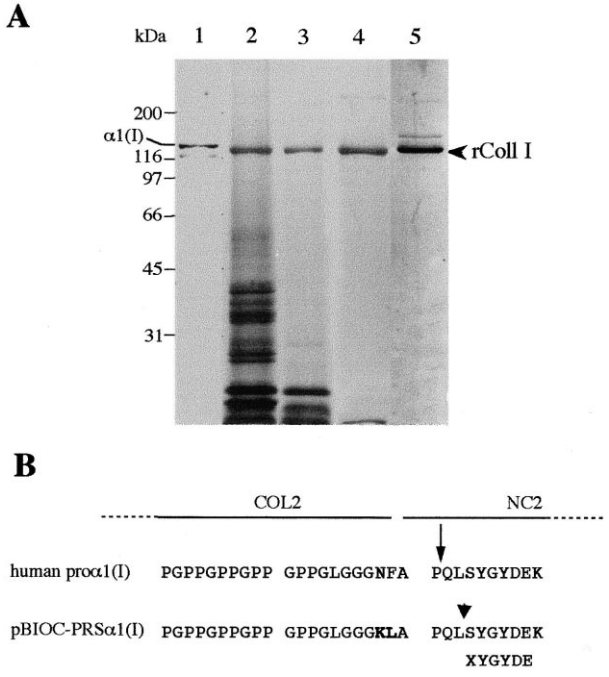

Fig. 3. A: Purification of recombinant collagen by salt fractionation of acid soluble crude extracts from pBIOC-PRS(ΔN-pro) (lanes 2–4) and pBIOC-PRSα1(I) (lane 5) mature transgenic plants. Samples were analyzed by 5–15% SDS–PAGE under reducing conditions and stained with Coomassie blue. Lane 1: purified bovine heterotrimeric collagen I. Lane 2: electrophoretic pattern of acid soluble crude transgenic tobacco extract. Lane 3: 0.4 M NaCl supernatant fraction. Lanes 4 and 5: 0.9 M NaCl precipitate containing purified recombinant collagen I (rColl I) from pBIOC-PRS(ΔN-pro) (lane 4) and from pBIOC-PRSα1(I) (lane 5). Left, running positions of protein standards are indicated in kDa. B: Comparison of the human α1(I) chain N-propeptide cleavage site (arrow) [17] with the N-propeptide cleavage site (arrowhead) of rColl I, purified from pBIOC-PRSα1(I) mature plants, determined by N-terminal amino acid sequencing (bottom line). Residue substitutions are bold face type; X, unidentified residue.

Table 1
Amino acid composition of recombinant collagen I

Amino acid	rColl I residues/ 100 residues	Bovine coll I homotrimer residues/100 residues
Hyp	0.53	9.04
Pro	18.75	10.32
Asp	4.87	4.66
Thr	1.67	1.91
Ser	4.74	3.79
Glu	8.87	10.13
Gly	33.85	33.53
Ala	10.49	9.74
Cys	ND	ND
Val	2.12	1.74
Met	0.56	0.70
Ile	0.69	0.80
Leu	2.28	2.65
Tyr	0.32	0.29
Phe	1.03	1.15
His	0.58	0.57
Hyl	0.08	1.63
Lys	4.13	2.46
Trp	ND	ND
Arg	4.66	4.96

ND, not determined.

mately 3 g of purified recombinant collagen is thus obtained per 100 kg of powdered plants allowing its molecular characterization.

As judged by the electrophoretic pattern, the major band detected in pBIOC-PRS(ΔN-pro) mature plant extracts nearly co-migrated with the pepsinized bovine α1(I) corresponding to the C-propeptide processed form (Fig. 3A, lanes 2–4). Two bands migrating at 140 kDa and 120 kDa, respectively, were detected in pBIOC-PRSα1(I) mature plant extracts, whereas the 160 kDa form, present in plantlet extracts, was not found. The intensity of the 140 kDa band, which corresponds in size to the C-terminal processed form, decreased during purification steps resulting in the increase of the 120 kDa band intensity. With time, the 120 kDa band became the major band detected in the purified sample (Fig. 3A, lane 5). This suggested that the recombinant product underwent further N-terminal proteolytic trimming during purification steps. Indeed, N-terminal sequencing indicated that a large part of the N-propeptide was removed. We determined the start at Ser-259 (Fig. 3B), two residues downstream of the natural cleavage site of N-proteinase which occurred at the Pro-256–Gln-259 bond [20]. Thus, as it occurs for the heterotrimeric collagen I extracted from tissues, the fully processed form retains about 20 amino acid residues at the N-terminal extremity referred to as the N-telopeptide (see Fig. 1). Consequently, whatever the construct used for plant transformation, spanning or not the complete N-propeptide, the final recombinant product is fully processed to collagen. This product is referred hereafter to as rColl I (Fig. 3A).

3.3. Molecular characterization of the recombinant collagen

Prolyl-hydroxylation of target residues within the collagenous domain is known to be critical for the formation and stability of the helix. Although plants do contain a prolyl 4-hydroxylase, its substrate specificity, even though not absolute, is characterized by the tetra (L-prolyl) sequence [21]. The plant enzyme cannot ensure the prolyl-hydroxylation of the collagenous triplet Xaa-Pro-Gly. Indeed, amino acid analysis showed that the proline residues of rColl I are not hydroxylated (Table 1), which correlates with the slightly faster migration of the recombinant α1(I) chains compared to the standard α1(I) chain migration (Fig. 3A). Hydroxylation was shown to be required for triple helix nucleation and propagation of the fibrillar collagen III [22]. Nevertheless, the spectrum obtained by circular dichroism for the recombinant protein (Fig. 4A) is typical of a triple helical conformation. In addition, we showed that purified rColl I is resistant to trypsin at room temperature, indicating that the recombinant homotrimeric α1(I) chains do form stable triple helices (Fig. 4C). The well-folded triple helical structure was also confirmed by rotary shadowing experiments which revealed the presence of the characteristic 300 nm long rod-like molecules in purified rColl I samples (Fig. 4D). These molecules present the same features in terms of length and shape as native collagen I molecules. Altogether, these data attest to the structural conformity of the collagen produced by the transformed tobacco with native collagen and show that, even though unhydroxylated, collagen chains can assemble into stable correctly folded triple helices in plant systems.

It has previously been reported that the thermal stability of collagens is directly related to the hydroxyproline content. The inhibition of hydroxylation by incubating cultured fibroblasts with α,α′-bipyridyl, an inhibitor of prolyl hydroxylase, resulted in the production of unhydroxylated collagen with a melting temperature of 24°C [23,24]. As a consequence of the

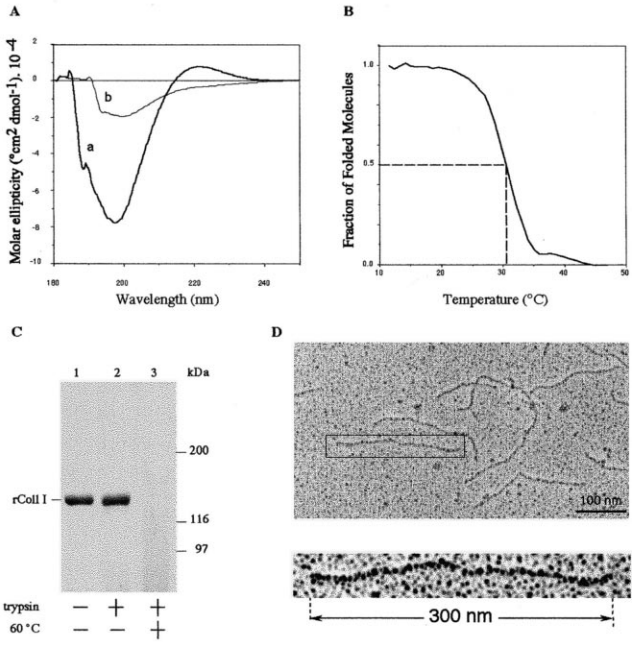

Fig. 4. Triple helix formation and thermal stability of purified rColl I molecules. A: Circular dichroism spectra of rColl I were recorded at 10°C (a) and at 53°C after denaturation (b). B: Thermal transition curve of rColl I as measured by circular dichroism at 225 nm. C: Formation of trypsin-resistant homotrimers. Purified rColl I was treated with (lane 2) or without (lane 1) trypsin at 25°C for 10 min at an enzyme/substrate ratio of 1:10. Positive control was achieved with heat-denatured (60°C for 20 min) rColl I samples (lane 3). Samples were analyzed by 6% SDS–PAGE under reducing conditions and stained with Coomassie blue. Right, molecular masses of protein standards are indicated in kDa. D: Rotary shadowed electron micrographs of purified recombinant homotrimers (rColl I). Representative field of recombinant molecules (top panel) and a selected (box) enlarged molecule (bottom panel) showing the characteristic 300 nm long rod-like collagen I molecule.

lack of prolyl-hydroxylation of the collagenous substrate in plant, it was thus expected that the thermal stability of our recombinant molecules would decrease compared to fully hydroxylated collagen. Indeed, the transition curves we obtained indicate that the recombinant collagen melts at 30.5°C (Fig. 4B) instead of 41.5°C for the homotrimer purified from bovine tissues (data not shown).

Interestingly, our results demonstrate that plants which a priori do not contain the specific post-translational equipment necessary for collagen molecule assembly guarantee the production of triple helical molecules. We have also showed that inter-chain disulfide bonds between the C-propeptides are formed as in animals. The folding and assembly of procollagen is a complex process and evidence is accumulating that suggests the involvement in chain selection and correct assembly of a collagen-specific molecular chaperone. The mammalian protein disulfide isomerase (PDI) was shown to bind to the C-propeptide prior to trimer assembly thereby also acting as a molecular chaperone during chain assembly [25]. Since PDI is present in tobacco plant cells [26], it might be involved in recombinant collagenous chain assembly.

Lastly, our data also show that plants provide a good system for producing fully processed molecules, which is a prerequisite for fibril formation. Spontaneous processing of procollagen to mature collagen has been shown to occur efficiently in tobacco plant extracts. This avoids the use of pepsin to cleave N- and C-terminal extensions which generally persist in recombinant collagen molecules obtained from other expression systems [4,5,7–10] including transgenic animals [11]. Moreover, electrophoretic patterns and N-terminal sequence analysis suggest that endogenous proteases cleaved at single sites within the N- and C-propeptides. N-propeptide cleavage occurs immediately downstream of the normal cleavage site of the N-proteinase. This result suggests that N- and C-propeptide cleavage can be ensured by enzymes other than the animal-specific enzymes, namely N-proteinase [20] and BMP-1 (C-proteinase) [27], presumably because the cleavage of both N- and C-propeptides occurs within the less folded regions that link two well-defined domains. Such regions might be more sensitive to proteases.

In conclusion, the data reported here demonstrate that collagen, a protein involved in a number of biomaterial and biotechnological applications, can be produced as fully processed triple helical molecules by transgenic tobacco plants. Although several other expression systems have been successfully developed for producing procollagens, the advantages of using plants include a high level expression of recombinant protein, a large-scale and cost-effective production as well as the avoidance of health risks in terms of viruses and infectious agents potentially present in other systems. The use of plants as bioreactors for collagen production makes it possible to produce unlimited quantities of other human collagens. The ease of co-expression in such a system also enables the production of heterotrimeric collagens as well as fully hydroxylated collagens by co-transformation in plants of cDNA encoding animal prolyl 4-hydroxylase.

In addition, our results have important implications for the development of genetically modified plants as sources of animal proteins. Indeed, the use of the tobacco plant for animal protein production potentially opens new possibilities for the exploitation of the already existing knowledge of the cultivation and processing of this plant in the tobacco industry.

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