Design and synthesis of heterotrimeric collagen peptides with a built-in cystine-knot

Models for collagen catabolism by matrix-metalloproteases

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Abstract **A heterotrimeric collagen peptide was designed and synthesized which contains the collagenase cleavage site (P_4-$P'_{9/10}$) of type I collagen linked to a C-terminal cystine-knot, and N-terminally extended with (Gly-Pro-Hyp)$_5$ triplets for stabilization of the triple-helical conformation. By employing a newly developed regioselective cysteine pairing strategy based exclusively on thiol disulfide exchange reactions, we succeeded in assembling in high yields and in a reproducible manner the triple-stranded cystine peptide. While the single chains showed no tendency to self-association into triple helices, the heterotrimer ($\alpha 1\alpha 2\alpha 1'$) was found to exhibit a typical collagen-like CD spectrum at room temperature and a melting temperature (T_m) of 33°C. This triple-helical collagen-like peptide is cleaved by the full-length human neutrophil collagenase (MMP-8) at a single locus fully confirming the correct raster of the heterotrimer. Its digestion proceeds at rates markedly higher than that of a single $\alpha 1'$ chain. In contrast, opposite digestion rates were measured with the catalytic Phe^{79}-MMP-8 domain of HNC. Moreover, the full-length enzyme exhibits K_m values of 5 μM and 1 mM for the heterotrimer and the single $\alpha 1'$ chain, respectively, which compare well with those reported for collagen type I (~1 μM), gelatine (~10 μM) and for octapeptides of the cleavage sequence (≥1 mM). The high affinity of the MMP-8 for the triple-helical heterotrimer and the fast digestion of this collagenous peptide confirm the decisive role of the hemopexin domain in recognition and possibly, partial unfolding of collagen.**

Key words: Collagen peptide; Synthesis; Heterotrimer; Triple helix; Enzymatic digestion; Collagenase; Catalytic domain; Hemopexin domain

1. Introduction

The structure of interstitial collagen consists of three extended left-handed poly(Pro)-II helices intertwined with a one-residue shift around a common axis to form a right-handed triple helix [1–4]. This triple-helical conformation makes interstitial collagen highly resistant toward all proteinases except specific collagenases (MMP-1, MMP-8 and MMP-13). They cleave all three strands of collagen at a single locus by hydrolyzing the peptide bond following the Gly residue of the partial sequences Gly-Ile-Ala (α1) and Gly-Leu-Leu (α2) located approximately three quarters from the amino-terminus. Sequence specificity is not sufficiently restrictive to account for the hydrolysis of native interstitial collagens at a single site as numerous potential cleavage sites are present in collagens. The lower content in Pro and Hyp, and thus the distribution of 'weak helix' triplets near the cleavage site [5] may direct its own proteolysis. Upon binding to full-length collagenases this local conformational softening of the triple helix may additionally be enhanced with the help of the hemopexin domain [6,7], Since triple-helical fragments are difficult to prepare by enzymatic cleavage of collagen, and generally were found to be thermally unstable [8,9], synthetic triple-helical model peptides containing the collagenase cleavage site could represent ideal tools to disclose the role of the hemopexin domain, and thus to study the mechanism of collagen catabolisms.

To overcome the unfavorable entropy of nucleating the triple helix in synthetic collagen-like peptides and to increase the thermal stability of the super-helix, covalent linkage of the peptide chains to templates such as 1,2,3-propanetricarboxylic acid or the Lys-Lys dipeptide, has been proposed [10–13]. The Lys-Lys template approach has extensively been exploited in the solid-phase synthesis of homotrimeric collagenous peptides [14] and even of a heterotrimer [15], despite the serious difficulties encountered in the synthesis and purification of such high molecular weight polypeptides. Since both these templates are highly flexible structures, the use of a conformationally constrained molecule, i.e. *cis,cis*-1,3,5-trimethylcyclohexane-1,3,5-tricarboxylic acid, has recently been examined by Goodman et al. [16]. It was found to be a highly efficient branching approach in terms of triple-helix induction and stabilization. For the formation of type III procollagen nature uses, possibly with the same intent, a cystine-knot which is located at the C-terminus of a collagenous subdomain and where each chain is linked to the other two chains by a complicated cystine framework [17].

For our de novo design of a trimeric collagenase substrate containing part of the molecule in a supposedly loose triple-helical, but nevertheless correctly rastered conformation, mimicking of this natural cystine-knot was attempted. Correspondingly, a structural model of a heterotrimeric collagenous triple-helical peptide was constructed where in a simplified cystine-knot the α2 chain was linked to both the α1 and α1′ chain by a cystine bridge (Fig. 1). The position of the cysteine residues in each chain was selected according to the structural model of collagen with (Gly-Pro-Hyp)$_n$ repeats [18]. The two disulfide bridges were fitted into this model according

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to the dihedral angle and bond length of a typical disulfide and to the distance of the two C-atoms of the cysteine residues [19]. Such built-in interstrand disulfide bridges were expected to stabilize the collagenous conformation and to constrain the heterotrimer into the correct raster. Therefore, the cystine-knot was placed at the C-terminus of the collagenase cleavage site of type I collagen [5], i.e. of the sequences P_4-$P'_{9/10}$ of the α1, α2 and α1′ chain, respectively. Since this portion of collagen should per se exhibit a very low tendency for triple-helical conformation, it was N-terminally extended with $(Gly\text{-}Pro\text{-}Hyp)_n$ triplets.

2. Materials and methods

2.1. Materials

All reagents and solvents used in the synthesis were of the highest quality commercially available. Human natural full-length neutrophil procollagenase (MMP-8) was prepared as described [20]. Its recombinant catalytic domain Phe^{79}-MMP-8 and the collagenase substrate Dnp-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2 were a gift of Dr. H.W. Krell, Boehringer-Mannheim (Penzberg).

2.2. Methods

Analytical RP-HPLC was carried out on Nucleosil 300/C8 (Machery and Nagel, Düren) using a linear gradient of acetonitrile/2% H_3PO_4 from 5:95 to 80:20 in 30 min and preparative RP-HPLC on Nucleosil 250/C18 with a linear gradient of acetonitrile (0.08% TFA)/0.1% TFA from 5:95 to 80:20 in 90 min. MALDI-TOF mass spectra were recorded on Bruker Reflex II instruments and amino acid analyses of the acid hydrolysates (6 M HCl containing 2.5% thioglycolic acid, 110°C; 72 h) were performed on a Biotronic analyzer (LC 6001).

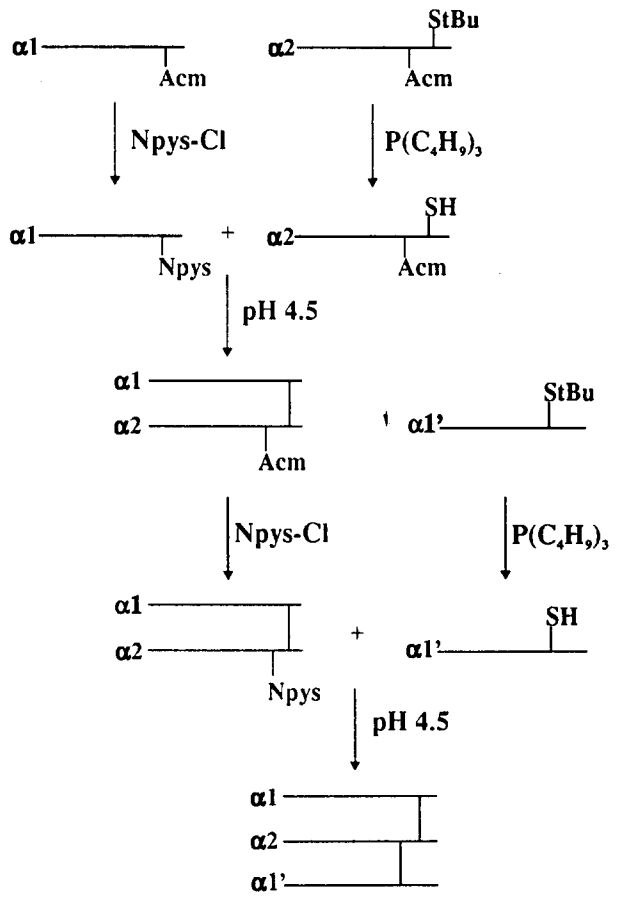
2.3. Synthesis of the heterotrimeric collagen peptides

A detailed description of the syntheses will be reported elsewhere. Briefly, the three chains α1[25,Cys(Acm)], α2[26,Cys(StBu),Cys(Acm)] and α1′[26,Cys(Acm)], suitably protected at the cysteine thiol functions, were synthesized on Wang resin using Fmoc/tBu protection [21] and HBTU/HOBt in the coupling steps [22] except for cysteine residues which were coupled as pentafluorophenyl esters to avoid racemization [23]. Cleavage from the resin was achieved with 95% aqueous TFA containing 1% triethylsilane. The three chains were assembled into the heterotrimer by stepwise regioselective crosslinking of the peptides, as outlined in Scheme 1, and careful purification (HPLC) and analytical characterization (HPLC, MALDI-TOF-MS) of all intermediates. The heterotrimer α1(25)α2(26)α1′(26) containing three Gly-Pro-Hyp repeats was obtained in 72% yield over all crosslinking reactions as a homogeneous compound (HPLC: $t_R = 14.43$ min); MALDI-TOF-MS: $m/z = 6824.9$ $[M]^+$; Calcd. for $C_{290}H_{484}N_{98}O_{85}S_4$: 6824.2; amino acid analysis: Glu 5.0 (5), Pro 11.28 (12), Gly 28.79 (30), Ala 3.05 (3), Cys 4.23 (4), Val 3.28(4), Ile 2.95 (3), Leu 4.08 (4), Arg 2.09 (2), Hyp 9.99 (10); peptide content: 76%.

Similarly, the heterotrimer α1(31)α2(32)α1′(32), containing five Gly-Pro-Hyp repeats, was assembled from the chains α1[31,Cys(Acm)], α2[32,Cys(StBu),Cys(Acm)] and α1′[32, Cys(StBu)] in 65% yield over the crosslinking steps; homogeneous in HPLC ($t_R = 14.20$ min); MALDI-TOF-MS: $m/z = 8427.6$ $[M]^+$; Calcd. for $C_{362}H_{586}N_{116}O_{105}S_4$: 8427.26; amino acid analysis: Glu 5.0, Pro 17.10 (18), Gly 24.98 (36), Ala 3.02 (3), Cys 4.30 (4), Val 3.05(4), Ile 2.97 (3), Leu 4.07 (4), Arg 2.11 (2), Hyp 15.78 (16); peptide content: 78%.

2.4. CD measurements

CD spectra were recorded on a Yobin-Yvon dichrograph Mark IV equipped with a thermostated cell holder and connected to a data station for signal averaging and processing. All spectra are averages of 10 scans and the spectra were recorded by employing quartz cells of 0.1 cm optical path length. The spectra are reported in terms of ellipticity units per mole of peptide residues ($[\Theta]_R$). The concentrations were determined by weight and peptide content as determined by quantitative amino acid analysis of the peptides. Solutions of the peptides (0.2 mg/ml) were prepared in the collagenase assay buffer (10 mM $CaCl_2$, 100 mM NaCl, 50 mM Tris-HCl, pH 7.5) and pre-equilibrated for 12 h in an ice-bath. Melting curves were determined by monitoring the changes in dichroic intensity at 221 nm using a heating rate of 0.3°C/min with a Lauda RKS thermostat.



Scheme 1. Regioselective disulfide bridging of the three chains α1[31,Cys(Acm)], α2[32,Cys(StBu),Cys(Acm)] and α1′[32,Cys(StBu)] by thiol disulfide exchange reactions to the heterotrimer α1(31)α2(32)α1′(32).

2.5. Collagenase assay

Native procollagenase (MMP-8) was activated by incubation in 10 mM $CaCl_2$, 100 mM NaCl, 50 mM Tris-HCl (pH 7.5) with 0.05 equivalents of trypsin at 37°C for 2 h; then 5 equivalents (relative to trypsin) of BPTI were added; under these conditions only partial activation of the procollagenase is achieved [24]. To a 90 μM solution of the heterotrimer α1(31)α2(32)α1′(32) or of the single α1′[26,Cys(Acm)] chain in 10 mM $CaCl_2$, 100 mM NaCl, 50 mM Tris-HCl (pH 7.5) at 25°C, aliquots of the stock solutions of the catalytic domain Phe^{79}-MMP-8 or of the activated full-length MMP-8 were added to reach concentrations of 8 and 0.4 nM, respectively. At given time intervals 20-μl aliquots of the assay solution were taken and quenched with 20 μl of 40% aqueous phosphoric acid. The quenched probes were analyzed by HPLC on Nucleosil 300/C8 using a linear gradient of acetonitrile/2% H_3PO_4 from 5:95 to 80:20 in 30 min.

Collagenase assays with the catalytic domain Phe^{79}-MMP-8 or with the activated full-length MMP-8 were performed at 25°C essentially according to the protocol of Stack and Gray [25]. Briefly, the fluorescent substrate Dnp-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2 (1×10^{-5} M) was used in 10 mM $CaCl_2$, 100 mM NaCl, 50 mM Tris-HCl (pH 7.5), and the increase in fluorescence at 346 nm was monitored over a period of 100 s to determine initial rates of hydrolysis. Since hydrolysis of the heterotrimer α1(31)α2(32)α1′(32) and the single chain α1′[26,Cys(Acm)] is negligible on this time scale, these peptides can be regarded as competitive inhibitors. Correspondingly, the related IC_{50} values, determined in this assay system, represent to a first approximation the K_m values.

3. Results

3.1 Synthesis of the heterotrimeric collagen peptides

Type I and type IV collagen are heterotrimers for which the contribution of the α2 chain toward structure and activity has not yet been examined [5]. Thus, synthetic triple-helical peptides containing one chain of different sequence from the other two would more realistically model the features of this type of natural collagens. The most promising approach for the assembly of such heterotrimers appeared to be the use of a simplified cystine-knot as outlined in Fig. 1. Various methods have been proposed in the past for the selective crosslinking of two cysteine peptides to a double-stranded asymmetric cystine peptide [26–30]. However, to the best of our knowledge, regioselective disulfide bridging of three cysteine-peptides has no yet been reported.

Because of the strong properties of self-association of collagenous peptides into homotrimers, difficulties were expected and encountered with the sulfenohydrazide procedure [27,28] already at the level of heterodimerization because of the facile oxidation of cysteine residues under the neutral or slightly basic conditions required for this method [31]. We have, therefore, examined for the present syntheses thiol disulfide exchange reactions that can be performed even under acidic conditions. As shown in Scheme 1, deprotection of the α1 Cys(Acm)] chain with *o*-nitropyridylsulfenyl chloride (Npys-Cl) [30], in analogy to the known procedures with other sulfenyl chlorides [26,32], leads to the activated disulfide which then undergoes in acidic media (pH 4.5) the thiol disulfide exchange reaction with α2[Cys(Acm),Cys] to produce the analytically well characterized heterodimer in over 80% yield. Subsequent removal of the Acm group from the dimer α1 α2[Cys(Acm)] with Npys-Cl and concomitant activation of the second cysteine residue of the α2 chain as an asymmetric disulfide allowed generation of the desired heterotrimer in a subsequent regioselective thiol disulfide exchange reaction with the cysteine-unprotected α1′ chain, again in nearly 80% yield over these two steps. Both heterotrimers, α1(25)-α2(26)α1′(26) and α1(31)α2(32)α1′(32) with 3 and 5 Gly-Pro-Hyp repeats, respectively, exhibited the correct mass in MALDI-TOF-MS and behaved homogeneously in HPLC.

3.2 Conformational properties of the heterotrimers

The collagenous triple-helical conformation is reflected by a characteristic CD spectrum with a relatively strong positive maximum at 221–224 nm and an intense negative maximum at 196–200 nm. Thereby, the ratio of the dichroic intensity of the positive peak over that of the negative peak (Rpn parameter), recently introduced by Feng et al. [33] and validated by

α1 H_2N-$(GPO)_5$GPQGIAGQRGVVGCGG-OH

α2 H_2N-$(GPO)_5$GPQGLLGAOGILGCCGG-OH

α1′ H_2N-$(GPO)_5$GPQGIAGQRGVVGLCGG-OH

Fig. 1. Sequences of a heterotrimeric collagenous peptide containing the collagenase cleavage site P_4-$P'_{9/10}$ of the α1, α2 and α1′ chain of type I collagen flanked at the C-terminus by an artificial cystine-knot and at the N-terminus by Gly-Pro-Hyp repeats known to induce and stabilize the triple helical conformation.

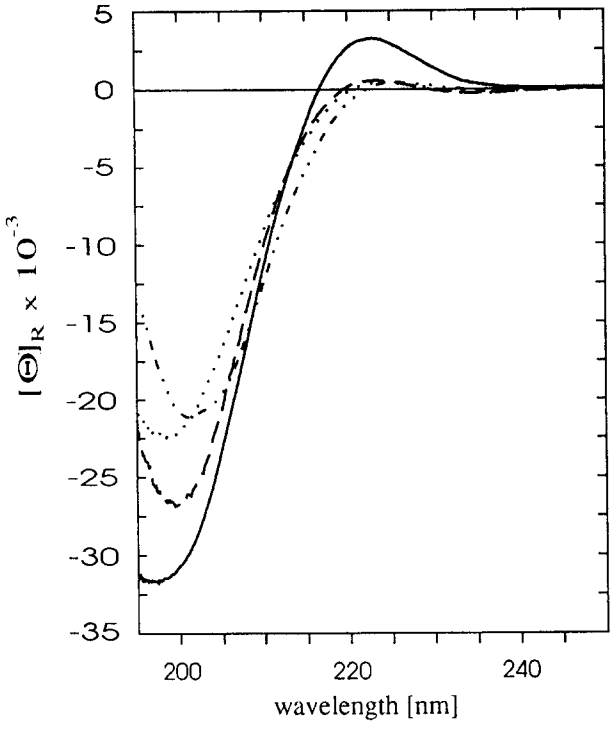


Fig. 2. CD spectra of α1′[26,Cys(Acm)] (- · · · -) at 1°C and of α1(25)α2(26)α1′(26) (– – –), α1′[32,Cys(StBu)] (· · ·) and α1(31)α2(32)α1′(32) (———) at 5°C in 10 mM $CaCl_2$, 100 mM NaCl, 50 mM Tris-HCl, pH 7.5; concentration: 0.2 mg/ml.

detailed NMR analyses [34], allows one to differentiate between the triple-helix conformation and poly(Pro)-II helix. Denaturation of the collagen triple helix occurs in a strongly cooperative manner with loss of the positive maximum and a red shift of the negative signal, thus allowing monitoring of thermal denaturation at 221 nm [35,36].

The heterotrimer α1(31)α2(32)α1′(32) with five Gly-Pro-Hyp repeats exhibits at 5°C the typical triple-helical CD spectrum with a positive maximum at 222 nm, a negative maximum at 196 nm and a crossover at 211 nm (Fig. 2). Its Rpn value of 0.12 at 5°C is fully consistent with a triple-helical fold and the melting curve is characterized by a sharp cooperative transition with a T_m of 33°C (Fig. 3). On cooling at rates identical to those of heating, renaturation occurs without hysteresis leading to an identical T_m value (33°C) and a CD spectrum at 5°C superimposable on that of the starting structure. The heterotrimer α1(25)α2(26)α1′(26) with three Gly-Pro-Hyp repeats still exhibits at 5°C the typical spectrum for the triple-helical conformation with an Rpn value of 0.12 and a crossover at 211 nm (Fig. 2); the related melting curve, however, is characterized by a significantly lower T_m value of 8–9°C (Fig. 3). Conversely, the single chain α1′[32,Cys(StBu)] with five Gly-Pro-Hyp repeats shows at 5°C a CD spectrum (Fig. 2) that could reflect partial triple-helical structure. However, its Rpn value of 0.022 clearly excludes a triple helix and indicates the presence of a poly(Pro)-II helix. This is further supported by the thermal denaturation curve with its non-sharp and thus poorly cooperative transition at $T_m = 20$°C (Fig. 3). Recooling of the solution and annealing of the system at 5°C for 20 min restores only partially the original CD spectrum. Finally, the CD spectrum of the single chain α1′[26,Cys(Acm)] with three Gly-Pro-Hyp triplets shows only at 1°C a collagen-type CD spectrum, how-

ever, with a Rpn value of 0.005 that again indicates a poly-(Pro)-II helix conformation (Fig. 2).

These results clearly confirm that covalent crosslinking of the chains via the cystine-knot largely reduces the entropic penalty of self-association and that upon extending the Gly-Pro-Hyp repeats from three to five the gain in conformational enthalpy is fully compensating the entropic cost in this folding process.

3.3. Proteolysis of the collagenous heterotrimers

On storage, active collagenases lose autolytically their C-terminal domain [37] which supposedly plays an important role in native collagenase activity. The resulting catalytic domain retains its proteolytic activity against denatured collagen, gelatin and single-stranded peptides, but is inactive against triple-helical collagen [37,38]. According to the CD spectrum the α1′[26,Cys(Acm)] chain is monomeric and denatured at 25°C. As expected, it is rapidly digested by the recombinant catalytic Phe^{79}-MMP-8 domain at this temperature into two peptides as monitored by HPLC (Fig. 4). Conversely, the heterotrimer α1(31)α2(32)α1′(32) is cleaved at very low rates confirming once more the triple-helical fold of this trimer at 25°C. Because of the known preference of full-length collagenases for native collagen, the trypsin-activated procollagenase showed a slow digestion of the single-stranded α1′[26,Cys(Acm)] and a fast cleavage rate for the heterotrimer α1(31)α2(32)α1′(32) (Fig. 5). By HPLC monitoring of the proteolytic products of the heterotrimer during the time course of enzymatic digestion, no intermediate products with one- or two-chain cleavages could be detected, as shown in Fig. 6. Thus, proteolysis must occur in a single cut of all three chains yielding only the single-stranded N-terminus and the C-terminal heterotrimer. This observation represents a strong hint as to the correct raster in the alignment of the three chains as induced by the artificial cystine-knot.

As both the single-stranded α1′[26,Cys(Acm)] chain and the heterotrimeric construct α1(31)α2(32)α1′(32) are digested at relatively low rates under the assay conditions used, the collagenous peptides can be considered as competitive inhibitors

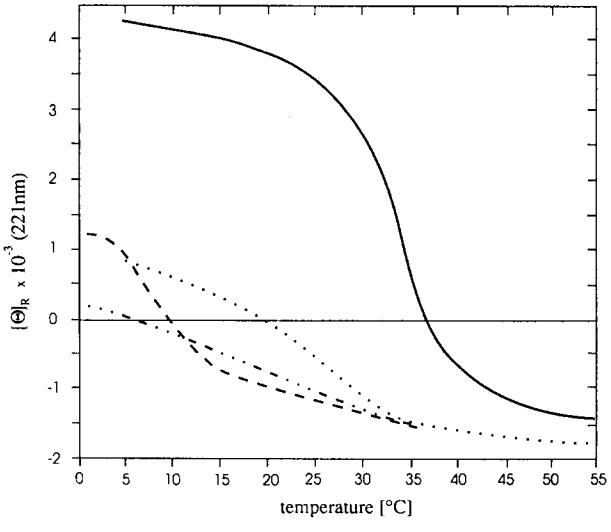


Fig. 3. Melting curves of α1′[26,Cys(Acm)] (- · · · -), α1(25)α2(26)α1′(26) (– – –), α1′[31,Cys(StBu)] (·····) and α1(31)α2(32)α1′(32) (——) in 10 mM $CaCl_2$, 100 mM NaCl, 50 mM Tris-HCl, pH 7.5; concentration: 0.2 mg/ml.

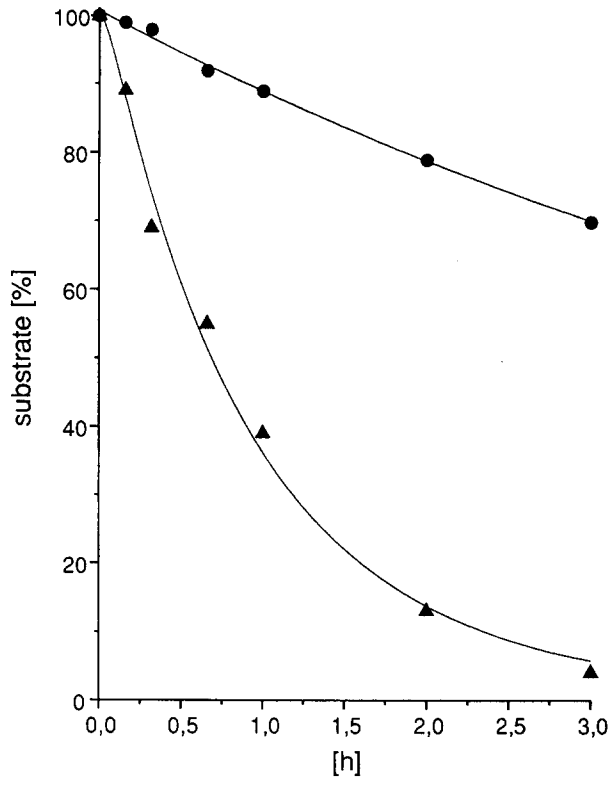


Fig. 4. Enzymatic digestion of α1′[26,Cys(Acm)] (▲-▲) and α1(31)α2(32)α1′(32) (●-●) by the catalytic domain of HNC (Phe^{79}-MMP-8) at 25°C and a substrate/enzyme ratio of 10000.

in the presence of the assay substrate Dnp-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2 and their IC_{50} values should correspond to a first approximation to the K_m values. The full-length collagenase (MMP-8) showed an IC_{50} ($\sim K_m$) of 5 μM for the heterotrimer and of 1 mM for the monomeric α1′[26,Cys(Acm)] chain. This dramatic difference again confirms the great preference of native collagenase for trimers in a collagenous conformation. In fact, it is known that triple-helical collagens have a very high affinity for full-length active collagenase with K_m values of ~1 μM, whereas K_m values of 10 μM and ≥1 mM were determined for gelatin and for octapeptides related to collagenase cleavage sequences, respectively [5].

4. Discussion

From previous studies on H-(Gly-Pro-Hyp)$_n$-OH it is known that with n=10, stable triple-helical homotrimers are formed at room temperature in aqueous solution [35], whereas in the case of Ac-(Gly-Pro-Hyp)$_n$-NH_2, i.e. by suppression of the endgroup effects, this triple helix is already induced with n=6 [33]. By additional crosslinking of the N-termini of the H-(Gly-Pro-Hyp)$_n$-NH_2 peptides with the conformationally constrained *cis,cis*-1,3,5-trimethylcyclohexane-1,3,5-tricarboxylic acid to the homotrimers, Goodman et al. [16] clearly showed a significant reduction of the number of tripeptide repeats required for a stable triple-helix conformation. With 3 repeats an incipient triple helix was identified by NMR analysis, whereas with 5 repeats a highly stable collagenous fold was obtained [33].

By flanking the large-size cleavage-site peptides of type I

collagen with a cystine-knot on one site and five Gly-Pro-Hyp repeats on the other site, we succeeded in markedly stabilizing a collagen-like structure although the amino acid sequence composition at the cleavage site is far from the ideal one for a triple-helix structure. This fact is probably due to a very good fit of the designed cystine-knot into the triple-helix structure, and thus to strong restraints imparted on the correct raster.

That this correct raster was achieved is confirmed not only by the thermal renaturation that occurs without hysteresis, but also by the single-cut cleavage of the heterotrimer into two components by the full-length MMP-8, i.e. containing the hemopexin domain. Most surprisingly, even the catalytic domain generates a single-cut cleavage although in a less efficient manner. The isolated catalytic domain of collagenase should preferentially cleave the unfolded heterotrimer, if partially present at conformational equilibrium, according to the observed rapid digestion of the single-stranded substrate by this truncated enzyme form. However, this processing of the unfolded substrate should lead to partially digested intermediates which could not be detected in the assay medium. Thus, the question remains as to how collagenases, apparently even their catalytic domains, retain the product of first digestion for further cleavage steps and what is then the role of the hemopexin domain in the dynamics of this proteolytic process.

With the new strategy of synthesis of collagenous heterotrimers presented in this communication, related analogs become more easily accessible and most importantly, the raster of the chains can be deliberately changed for a detailed study of the mechanism of proteolysis of interstitial collagens by the matrix-metalloproteases.

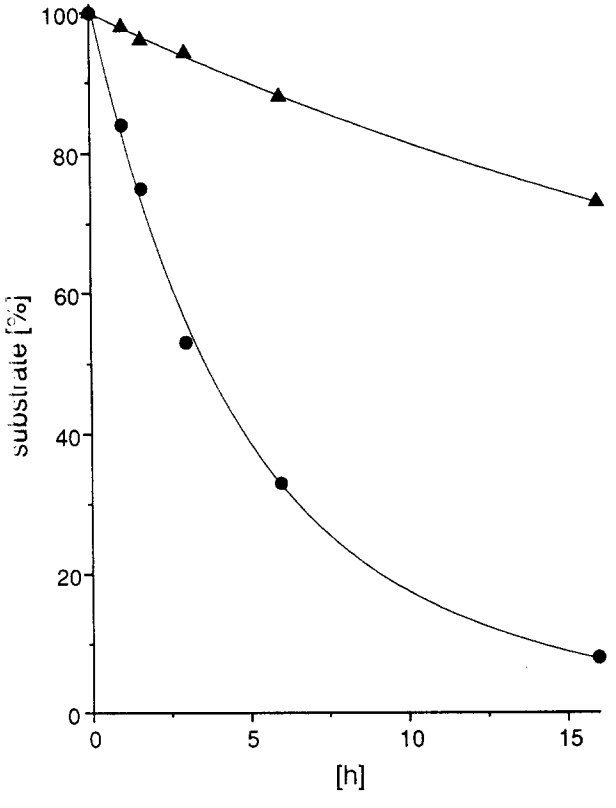


Fig. 5. Enzymatic digestion of α1′[26,Cys(Acm)] (▲-▲) and α1(31)α2(32)α1′(32) (●-●) by the full-length HNC at 25°C and a substrate/ enzyme ratio of ~100000.

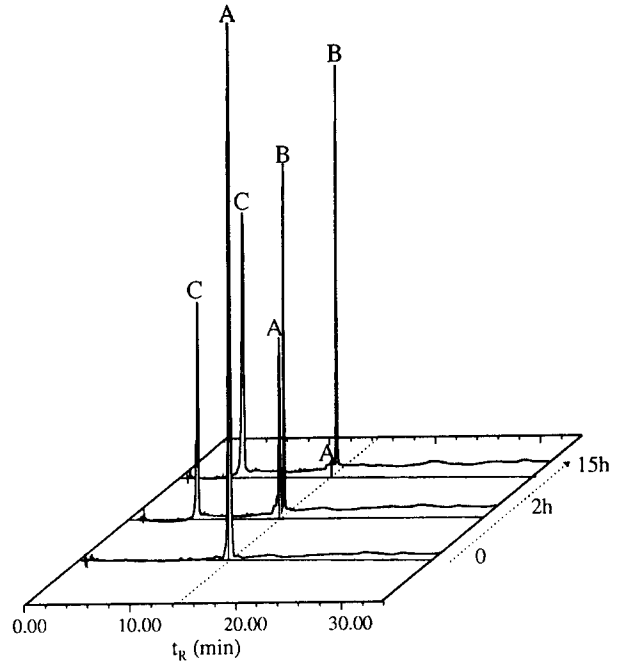


Fig. 6. HPLC patterns of the enzymatic digestion of α1(31)α2(32)α1′(32) by the full-length HNC at 25°C at different time intervals; (A) trimeric substrate; (B,C) cleavage products.

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