Journal of Peptide Science
J. Pept. Sci. 2007; **13**: 121–127
Published online 5 December 2006 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.823

JOURNAL OF PEPTIDE SCIENCE

Collagen models as a probe in the decay of works of art: synthesis, conformation and immunological studies

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Received 7 July 2006; Revised 4 October 2006; Accepted 17 October 2006

Abstract: Proteinaceous substances such as collagen, casein and albumin have been widely used as binding media in a variety of works of art. Damages of these 'sensitive' materials, mainly caused of the influence of the environment, are responsible for the overall decay of works of art, and their identification is essential to understand ancient technologies, determine the extent of deterioration and help in future restoration and preservation processes. The most commonly used techniques for the identification of proteinaceous binding media are staining techniques, chromatography, spectrometry and immunological methods, although for the latter no systematic studies have been carried out until now. Aiming at contributing to the development of a reliable and reproducible immunoassay for the evaluation of the collagen-based decay of works of art, sequential polypeptides $(Pro\text{-}X\text{-}Gly)_n$ where X represents amino acid residues Val, Lys, Glu and $(Hyp\text{-}Val\text{-}Gly)_n$ were prepared as models of collagen fragments derived from artificially and naturally aged animal collagens. Conformational studies of the polypeptides by CD revealed the occurrence of polyproline II-like structures comparable with those of collagen. Polypeptides and collagen I were administered to animals, and the induced antibodies were used for the immunochemical detection and differentiation of collagen and collagen fragments. The combined application of (i) anti-collagen antibodies, which strongly interact with native collagen, but poorly recognized by artificially aged collagen and (ii) anti-polypeptide antibodies, which do not associate with native collagen, but are strongly recognized by collagen fragments in naturally or artificially aged collagen, is a valuable tool in determining the extent of decay in works of art. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: collagen model peptides; circular dichroism; immunological assays; artificial ageing of collagen; decay of works of art

INTRODUCTION

The importance of preserving cultural heritage all over the world need not be stressed upon, as it coincides with the very existence of our civilization. Preservation of cultural heritage, once done in a purely empirical manner, often poorly documented, is nowadays the subject of thorough examination and the scientific rationale for conservation practices is actively pursued. As a result, the tools for diagnosis of the deterioration status, due to physical, chemical and biological damages, and the methods employed in restoration and conservation are using state-of-the-art technology and science.

Proteinaceous substances (such as collagen, casein, egg yolk, egg white, albumin and hemoglobin) have been widely used as binding media in a variety of works of art such as paintings, stone sculptures and wooden statues. Damages of these 'sensitive' proteinaceous materials, mainly caused by the influence of the environment, temperature, humidity, air pollutants and light, are responsible for the overall damage of works of art. Identification of proteins is essential to understand ancient technologies, determine the extent of decay and help in future restoration and preservation processes. The most commonly used techniques for the identification of proteinaceous binding media are staining techniques, as well as chromatography and spectrometry [1–4].

Immunological techniques such as radio-immunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA) have been proposed as an alternative method for this purpose [5–7], although no systematic studies have been carried out until now. These methods have several potential advantages for the analysis of paint media over those mentioned above and they are very promising in the field of conservation of works of art. Their main advantages are sensitivity to nanogram quantities of material, high specificity of the reaction which makes it possible to distinguish between two different proteins, as well as the same protein from different species, easier interpretation of results in samples containing mixtures of proteins, submicron resolution in spatial arrangement of materials, tolerance to a certain amount of structural alterations in their target antigens and application in a wide variety of materials.

A variety of immunological studies have been reported in the literature for the detection of collagen, hemoglobin and albumin in archaeological bones

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using RIA and ELISA tests [8–11]. Fluorescent-labelled antibodies have been used to localize different collagen types in archaeological bones [12], while immunofluorescence techniques have also been used to identify the media of 15th–18th century European paintings [5]. Recently the immunological detection of artificially aged collagen paints by different types of antibodies was reported [6].

Collagen has been widely used as a binding medium in works of art. The tertiary structure of collagen is a triple helix, which consists of three left-handed polyproline II-like chains intertwined to form a right-handed triple helix [13–15]. The primary structure of the peptide chains is characterized by repeating trimer units Gly-X-Y, where X and Y are frequently proline (Pro) and 4-hydroxyproline (Hyp) and account for about 20% of the residues in collagen sequences [16,17]. Other frequently occurring amino acids in collagen include Ala, Lys, Arg, Leu, Val, Phe and Glu [18,19].

The use of synthetic polypeptide models has proved a valuable tool for the study of the structure and stability of collagen triple helix, as well as in understanding the biological function of collagen. Synthetic collagen models are used as an alternative source of new materials for a wide variety of applications. Many sequential polypeptides composed of Gly-X-Y sequences have been synthesized by polymerization of the active esters of the appropriate tripeptides to mimic collagen-like triple helical structures [20–24]. The development of solid-phase peptide synthesis (SPPS) has allowed the synthesis of sequential oligopeptides of varying sequences and chain lengths, by fragment coupling of Gly-X-Y tripeptides that self-associate to form triple helical conformations [25–35]. Template-assembled collagen-based polypeptides have also been synthesized by the solid-phase segment condensation methods [36].

With the aim of further contributing to the development of a reliable and reproducible immunoassay for the evaluation of the collagen-based deterioration of works of art, we report now the design, synthesis and conformational and immunological study of a series of sequential polypeptides as models of collagen fragments derived from artificially and naturally aged animal collagens. Sequential polypeptides $(\text{Pro-X-Gly})_n$ where X represents amino acid residues Val, Lys, Glu and $(\text{Hyp-Val-Gly})_n$ were prepared and their conformational properties were evaluated by circular dichroism (CD) studies in aqueous and organic mixtures and compared with type I collagen from calf skin. Antibodies induced in animals injected with collagen I and synthetic polypeptides were used for the immunochemical detection and differentiation of collagen and collagen fragments in artificially and naturally aged samples to determine the extent of decay. Our objective is to provide a reference ELISA as a diagnostic tool for the assessment of the decay in artworks and possibly new insights into the preservation of cultural heritage.

MATERIALS AND METHODS

Peptide Synthesis

Synthesis of the protected tripeptides Boc-Pro-AA$_2$-Gly-OH, AA$_2$ = Val, Lys(Alloc), Glu(OAll). The synthesis of the protected tripeptides was carried out by the SPPS [37–39] on a 2-chlorotrityl-chloride resin (1.35 mmolCl^-/g resin) using the Fmoc methodology. Lysine and glutamic acid were introduced as Fmoc-Lys(Alloc)-OH and Fmoc-Glu(OAll)-OH, respectively. Fmoc groups were removed using 20% piperidine in DMF. The coupling reactions were performed using an amino acid/TBTU/HOBt/DIEA/resin molar ratio of 3/3/3/9/1 (TBTU: *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetra-methyluronium tetra-fluoroborate, HOBt: 1-hydroxybenzotriazole, DIEA: *N*,*N*-diisopropylethylamine). DMF, used for couplings, was distilled in the presence of ninhydrin to remove traces of amines. Completion of the coupling reactions was ensured by the use of the Kaiser ninhydrin test. The protected tripeptides were cleaved from the resin by treatment with a mixture of acetic acid/trifluoroethanol/dichloromethane (2/2/6, v/v/v). The resin was removed by filtration, the filtrate was evaporated under reduced pressure and the product was precipitated with cold diethyl ether. Yields after recrystallization (methyl alcohol/diethyl ether) ranged from 70 to 80%. The purity of the peptides was checked by analytical HPLC and the correct molecular masses were confirmed by ESI-MS:

Boc-Pro-Val-Gly-OH: expected $M^- = 370.43$, found $M^- = 370.48$
Boc-Pro-Lys(Alloc)-Gly-OH: expected $M^- = 483.54$, found $M^- = 483.72$
Boc-Pro-Glu(OAll)-Gly-OH: expected $M^- = 440.47$, found $M^- = 440.53$

Synthesis of the TFA.Pro-AA$_2$-Gly-Opcp, AA$_2$ = Val, Lys(Alloc), Glu(OAll). To a solution of the appropriate Boc tripeptide unit (1 mmol) and pentachlorophenol (1.1 mmol) in dioxane, dicyclohexylcarbodiimide (DCC) (1.1 mmol) in dioxane was slowly added, and after 24 h the reaction mixture was filtrated and lyophilized (yields ranged from 80 to 90%). Boc deprotection was achieved with anhydrous trifluoroacetic acid (TFA) for 30 min, the solution was evaporated *in vacuo* and the residue was precipitated with anhydrous ether. The correct molecular masses were confirmed by ESI-MS:

Pro-Val-Gly-Opcp: expected $M^+ = 520.65$, found $M^+ = 520.14$
Pro-Lys(Alloc)-Gly-Opcp: expected $M^+ = 633.76$, found $M^+ = 633.49$
Pro-Glu(OAll)-Gly-Opcp: expected $M^+ = 590.69$, found $M^+ = 590.46$

Synthesis of the polypeptides (Pro-X-Gly)$_n$ X=Val, Lys, Glu. Polymerization of the pentachlorophenyl esters of the tripeptide units was carried out in DMF in the presence of triethylamine for 8 days [40]. DMF was evaporated *in vacuo* and the product precipitated with anhydrous ether (yields

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DOI: 10.1002/psc

Table 1 Solution polymerization of tripeptide pentachlorophenyl esters

Starting material	Polymer	nsp/c[a]	Average molecular weight	Amino acid analysis
TFA.Pro-Val-Gly-Opcp	Poly(Pro-Val-Gly)	0.19	28 000	Pro: Val : Gly 1 : 1:1
TFA.Hyp-Val-Gly-Opcp	Poly(Hyp-Val-Gly)	0.19	28 000	Hyp : Val : Gly 1 : 0.8 : 0.8
TFA.Pro-Lys(Alloc)-Gly-Opcp	Poly(Pro-Lys-Gly)	0.20	30 000	Pro: Lys : Gly 0.8 : 1 : 1
TFA.Pro-Glu(OAll)-Gly-Opcp	Poly(Pro-Glu-Gly)	0.17	25 000	Pro: Glu : Gly 0.9 : 1 : 1.1

[a] Polymer concentration 0.4g/100 ml dichloroacetic acid. nsp/c stands for intrinsic viscosity.

ranged from 50 to 60%). The Alloc and Allyl protecting groups were removed using the hydrostannolytic cleavage [41]. The polymer (1 mmol), $PdCl_2(PPh_3)_2$ (0.02 mmol) and acetic acid (2.4 mmol) were dissolved in 10 ml CH_2Cl_2, and 1.1 mmol of Bu_3SnH was rapidly added with a syringe, to the stirred solution. A slightly exothermic reaction with vigorous gas evolution immediately ensued. The solution was evaporated *in vacuo* and the residue precipitated with anhydrous ether. The polymers were dialysed using a 1200 molecular weight cut-off dialysis tubing against water and lyophilized. Amino acid analysis of the polymers gave the expected ratio: poly(Pro-Val-Gly) Pro: Val : Gly 1: 1 : 1, poly(Pro-Lys-Gly) Pro: Lys : Gly 0.8 : 1 : 1, poly(Pro-Glu-Gly), Pro: Glu : Gly 0.9 : 1 : 1.1. The average molecular weight of the polymers was estimated, using viscosity measurements in dichloroacetic acid, to be 25 000–30 000 (Table 1).

Synthesis of the polypeptide (Hyp-Val-Gly)$_n$. The mixed anhydride procedure was used for all couplings, with sufficient yield and purity. To a solution of Boc-Val-OH (1.1 mmol) and *N*-methyl-morpholine (1.1 mmol) in anhydrous tetrahydrofuran, isobutyl chlorophormate (1.1 mmol) was added at −17 °C, followed after 5 min by a solution of glycine methyl ester hydrochloride (1 mM) and triethylamine (1 mM) in tetrahydrofuran, and after 24 h at room temperature the reaction mixture was evaporated to dryness. The residue was dissolved in ethyl acetate, washed with 1 M $NaHSO_4$, 1 M $NaHCO_3$ and water, evaporated *in vacuo* and the product precipitated with hexane (yield 81%). The Boc group was removed by anhydrous trifluoroacetic acid for 30 min, the solution was evaporated *in vacuo* and the product was precipitated with anhydrous ether. The synthesis of the Boc-Hyp-Val-Gly-OMe was carried out by the mixed anhydride method as described above (yield 65%). The glycine methyl ester was removed by saponification with 1N NaOH in dioxane, and the pentachlorophenyl ester was used for the carboxyl activation. To a solution of Boc-Hyp-Val-Gly-OH (1 mmol) and pentachlorophenol (1.1 mmol) in dioxane, DCC (1.1 mmol) in dioxane was slowly added and after 24 h the reaction mixture was filtered and lyophilized (yield 85%). The Boc deprotection was achieved with anhydrous trifluoroacetic acid for 30 min.

The polymerization of the pentachlorophenyl ester of the tripeptide unit was carried out in DMF in the presence of triethylamine for 8 days. DMF was evaporated *in vacuo* and the product precipitated with anhydrous ether (yield 60%). The polymer was dialysed using a 1200 molecular weight cut-off dialysis tubing against water and lyophilized. Amino acid analysis of the polymer gave the expected ratio, Hyp : Val : Gly 1 : 0.8 : 0.8. The average molecular weight of the polymer was estimated, using viscosity measurements in dichloroacetic acid, to be 28 000 (Table 1).

Biological Assays

Rabbit Immunizations – ELISA experiments. Polypeptides and calf skin collagen I (Sigma) were used to immunize New Zealand white rabbits. The animals received, first, an injection of 1mg of peptide in 500 µl H_2O and 500 µl complete Freund's adjuvant and then repeated injections of 0.5 mg of peptide in 500 µl H_2O and 500 µl incomplete Freund's adjuvant. Blood was collected from the ear of each animal 7 days after each injection. The collected sera were tested for the presence of antibodies against the synthetic polypeptides and collagen by ELISA [42,43] according to the following protocol: Synthetic polypeptides (5 µg/ml), as well as collagen (1 µg/ml) in $Na_2CO_3/NaHCO_3$ buffer, pH 9.6, were coated on 96-well polystyrene plates (100 µl/well) and incubated overnight at 4 °C. After washing with PBS buffer, pH 7.2, the non-specific binding sites were blocked with 3% skimmed milk in PBS and the plates were incubated at room temperature for 2 h. Serum samples from the pre-immunized and post-immunized animals at dilutions, with blocking buffer, from 1 : 100 to 1 : 800, were added (100 µl/well) and the plates incubated at 4 °C overnight. After extensive washings with PBS, the wells were incubated with goat anti-rabbit IgG conjugated to peroxidase (dilution 1 : 2000 with blocking buffer) at 37 °C for 1.5 h. Finally the plates were washed with PBS buffer, and 50 µl of substrate solution of TMB (3,3′,5, 5′ tetramethyl-benzidine, 1/1 v/v H_2O) and 50 µl of H_2O_2 (1/1 v/v H_2O) were added to each well, and the absorbance was measured at 450 nm.

Artificial Ageing of Collagen

Known quantities of collagen type I (Sigma) were coated on glass slides, air dried, placed in the Fadeometer (Climate-Test-Cabinet model 4001, RUMED Rubarth Apparate GmbH, Laatzen, Germany), and subjected to artificial ageing at 40 °C and 80% relative humidity. Slides were removed periodically and samples were extracted by an aqueous solution of 1% NH_3, lyophilized and stored at −20 °C until analysis.

Naturally Aged Samples

Naturally aged collagen samples were taken from the mortar of a 16th century wall painting from the I.M. Dionysios monastery, Holly Mountain Athos in Greece and from archaeological and contemporary human bones. Collagen and collagen fragments were obtained from naturally aged samples by treatment with aqueous solution of 1% NH_3. The solution was lyophilized and the product was diluted with water, in order to prepare a stock solution of 1mg/ml, and stored at −20 °C until analysis.

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DOI: 10.1002/psc

Circular Dichroism

CD spectra were recorded on a Jasco J-710 spectropolarimeter equipped with a thermoelectric temperature controller. Spectra were obtained using a quartz cell of 1 cm path length by signal-averaging 3 scans, from 180 to 260 nm, with a scan speed of 50 nm/min, and were smoothed after subtraction of the solvent spectrum. Polymer concentrations of 0.05–0.2 mg/ml in H_2O and ethyleneglycol/H_2O, v/v 2 : 1 were used to obtain the CD spectra at 20 °C and pH 7. All CD spectra are reported in terms of ellipticity units per mole of peptide residue [Θ] in deg cm^2 $dmol^{-1}$. The CDPro calculation program was used for analyzing the CD spectra of all polymers in different environments.

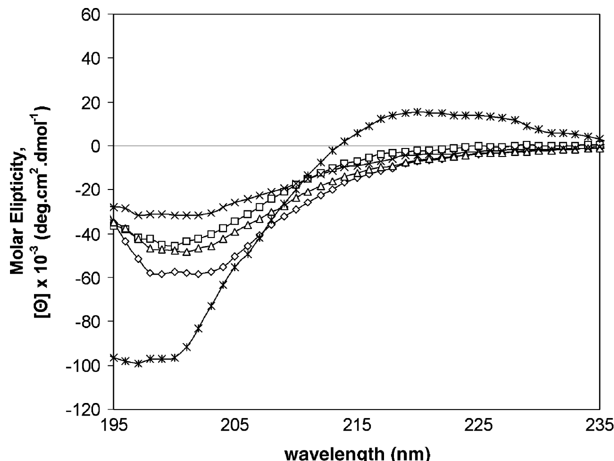

Figure 1 CD spectra of polypeptides in water: ×, poly(Pro-Val-Gly), 0.15 mg/ml; □, poly(Hyp-Val-Gly), 0.15 mg/ml; Δ, poly(Pro-Lys-Gly), 0.15 mg/ml; ◊, poly(Pro-Glu-Gly); and *, collagen 0.05 mg/ml.

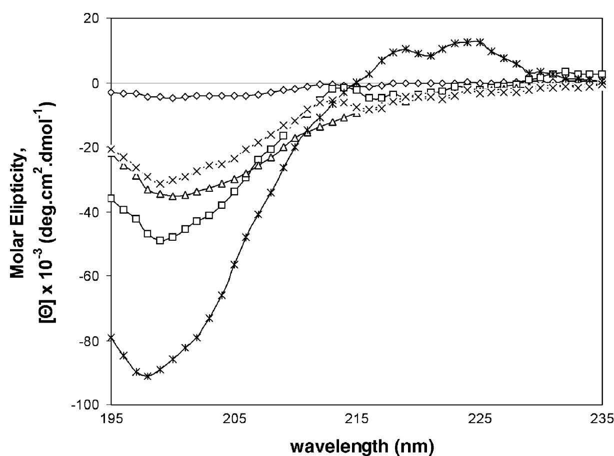

Figure 2 CD spectra of polypeptides in ethylene glycol/H_2O, v/v 2 : 1: ×, poly(Pro-Val-Gly), 0.042 mg/ml; □, poly(Hyp-Val-Gly), 0.042 mg/ml; Δ, poly(Pro-Lys-Gly), 0.25 mg/ml; ◊, poly(Pro-Glu-Gly), 0.25 mg/ml; and *, collagen 0.05 mg/ml.

RESULTS AND DISCUSSION

Conformational Study by CD

The natural collagen triple helix exhibits a unique CD spectrum with a small positive peak around 220 nm and a large negative peak around 197 nm [20,21,44]. These characteristic bands have also been found in polyproline II-like structures in aqueous mixtures with ethylene glycol (EG), which is known to stabilize triple helical structures and has been widely used to detect triple helical conformations of synthetic collagen models [26–28,35,45]. The CD spectra of collagen type I in water and EG/H_2O (v/v 2 : 1), shown in Figures 1 and 2, exhibit a small, broad, positive band at 225 nm and a large negative band at 200 nm typical of triple helix, and the percentage of polyproline II content of collagen was estimated by the CDPro calculation program to be 23% and 18% in water and EG/H_2O (2 : 1), respectively. A negative CD band is observed at 200 nm, comparable with that of collagen type I, for poly(Pro-Val-Gly), poly(Hyp-Val-Gly), poly(Pro-Lys-Gly) and poly(Pro-Glu-Gly) in water and EG/H_2O (v/v, 2 : 1), while the broad positive band at 220 nm is absent (Figures 1 and 2). Analysis of the CD spectra, by the CDPro calculation program, estimated the polyproline II percentage of all the polypeptides to be ∼15%, which is similar to that in collagen I in EG/H_2O (2 : 1).

Serological Study

Antibodies against collagen and collagen models. Sera were collected before the initial immunization, as well as 7 days after each boost, and were tested for the presence of antibodies against the immunizing antigens by ELISA. The reactivity of rabbit antisera to the calf skin collagen and polypeptides increased gradually and remained at high levels after the fifth immunization. Specific antibodies recognizing the priming immunogens were produced in all immunizing experiments. Antibodies raised against poly(Pro-Val-Gly), which exhibited the best persistence, were further used together with anti-collagen antibodies for the immunochemical detection of collagen and collagen fragments in artificially and naturally aged samples. Dilution curves of sera in anti-collagen and anti-poly(Pro-Val-Gly) ELISA are shown in Figures 3 and 4 respectively.

The type of the antigen tertiary structures recognized by the produced antibodies was tested in an ELISA experiment shown in Figure 5. Anti-collagen antibodies are highly recognized by collagen and to a lesser extent by anti-poly(Pro-Val-Gly) antibodies (see also Figure 6, S1). On the contrary, antibodies raised against poly(Pro-Val-Gly) strongly interact with the priming polypeptide and to a lesser degree with collagen. These results, in combination with the CD data, support that anti-oligomer antibodies recognize single helices, while anti-collagen antibodies recognize triple helices. The

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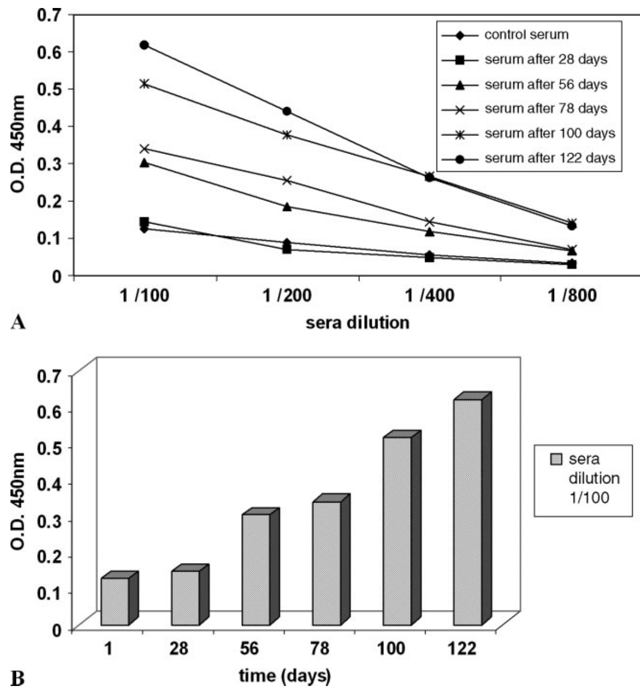

Figure 3 Binding of rabbit antisera to the calf skin collagen at dilutions from 1 : 100 to 1 : 800 (A). Comparison of antisera at 1 : 100 dilution at different time points from 1 to 122 days (B). Control serum : preimmune.

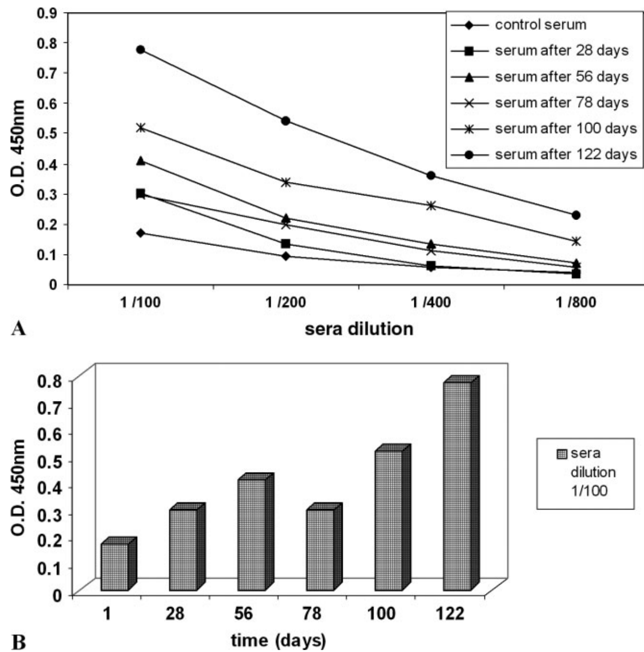

Figure 4 Binding of rabbit antisera to the poly(Pro-Val-Gly) at dilutions from 1 : 100 to 1 : 800 (A). Comparison of antisera reactivity at 1 : 100 dilution at different time points from 1–122 days (B). Control serum : preimmune.

observed partial cross-reactivity can be explained by the fact that both collagen and poly(Pro-Val-Gly) display approximately the same percentage of polyproline II as defined by CD.

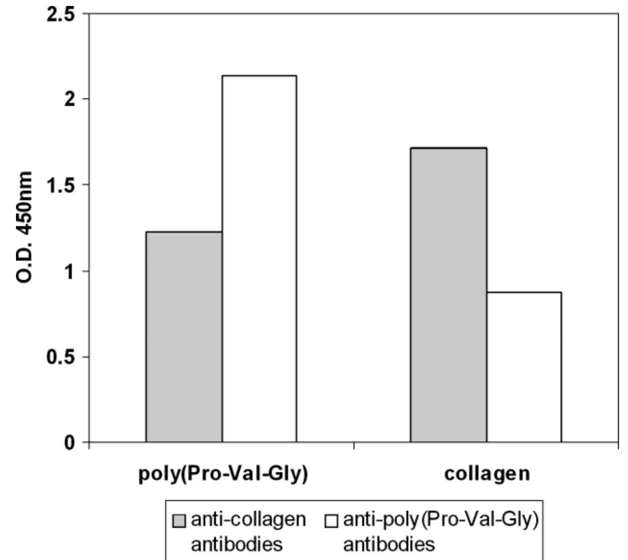

Figure 5 Binding of anti-collagen and anti-poly(Pro-Val-Gly) antibodies to poly(Pro-Val-Gly) and collagen. Sera dilution 1/100.

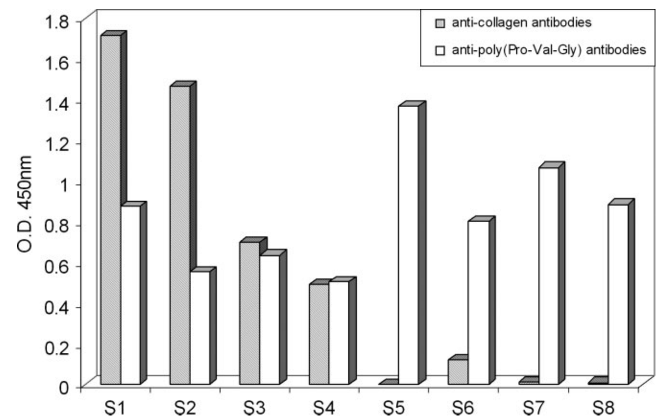

Figure 6 Binding of anti-collagen and anti-poly(Pro-Val-Gly) antibodies to artificially and naturally aged samples. S1: collagen type I, 0 days (control); S2: 31 days ageing; S3: 76 days ageing; S4: 175 days ageing; S5: artificially aged animal glue (rabbit glue) and gesso ($CaSO_4.2H_2O$); S6: sample of wall painting (I.M. Dionysios monastery, Holly Mountain); S7: collagen from archaeological human bones; S8: collagen from contemporary human bones.

Immunochemical Detection of Collagen and Collagen Fragments in Artificially and Naturally Aged Samples

Anti-collagen and anti-poly(Pro-Val-Gly) antibodies were used in order to determine the extent of decay of collagen in artificially aged samples, as well as in authentic samples, such as archaeological bones and wall paintings from I.M. Dionysios monastery, Holly Mountain, Athos, Greece. The ELISA experiments, shown in Figure 6, indicate the following: (i) The artificially aged collagen samples are highly recognized by the anti-collagen antibodies and to a lesser degree

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DOI: 10.1002/psc

by the anti-poly(Pro-Val-Gly) antibodies after 30 days in Fadeometer (S2). Anti-collagen antibody recognition is considerably decreased until 175 days (S3, S4), while recognition by the anti-poly(Pro-Val-Gly) antibodies remains relatively unchanged. It is assumed that the ageing conditions (temperature 40 °C, relative humidity 80%, time period 175 days) caused denaturation of collagen to some extent, resulting in a decrease of its recognition by the anti-collagen antibodies, while association with anti-poly(Pro-Val-Gly) antibodies remained stable as collagen was not hydrolysed to give fragments recognized by these antibodies. (ii) Sample S5, which was subjected to more drastic ageing conditions, is not recognized at all by the anti-collagen antibodies, while it is highly interacting with the anti-poly(Pro-Val-Gly) antibodies. (iii) Naturally aged collagen samples S6, S7, S8 are not recognized by the anti-collagen antibodies, while they are strongly interacting with anti-poly(Pro-Val-Gly) owing to the significant hydrolysis of collagen to fragments possibly similar to the polypeptide.

It is concluded that increase in the extent of collagen decay decreases the anti-collagen antibody recognition and enhances the anti-poly(Pro-Val-Gly) antibody interaction. These findings suggest the utility of anti-collagen and anti-poly(Pro-Val-Gly) antibodies in determining the extent of decay of collagen in works of art.

CONCLUSIONS

Sequential polypeptides $(\text{Pro-X-Gly})_n$, X = Val, Lys, Glu and $(\text{Hyp-Val-Gly})_n$ with molecular weights 25 000–30 000 were prepared as models of collagen. Conformational studies of these polypeptides revealed the occurrence of polyproline II-like structures in percentage comparable to that of collagen although they do not adopt the typical collagen triple helix.

The immunochemical study of antibodies raised in rabbits against collagen and poly(Pro-Val-Gly) indicated the occurrence of two types of antibodies: (i) anti-collagen antibodies that strongly interact with native collagen, while not recognizing, or recognizing to a lower degree, naturally or artificially aged collagen depending on the extent of its decay; and (ii) anti-poly(Pro-Val-Gly) antibodies that do not associate with native collagen, while strongly recognizing collagen fragments in naturally or artificially aged collagen.

It is concluded that the combined application of two types of antibodies, anti-collagen and anti-poly(Pro-Val-Gly), is a valuable tool in determining the extent of deterioration of collagen in works of art. The concept of our approach is also expected to provide new insights into future restoration and preservation processes.

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DOI: 10.1002/psc