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Study of the degradation of gelatin in paper upon aging using aqueous size-exclusion chromatography

Anne-Laurence Dupont*

National Gallery of Art, Conservation Division, Scientific Research Department, Washington, DC 20565, USA

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Abstract

We studied the aging behaviour of gelatin used to size paper. Thus far, research on the aging of paper has largely ignored the sizing agent. Degradation of the protein was characterised and the impact of paper components, such as cellulose, and aluminium potassium sulphate was evaluated. Whatman No. 1 filter papers sized with two types of gelatins (A and B) were prepared as model samples. Commercially sized modern papers (Arches) were also studied in order to compare laboratory samples with real artist papers. Both types of papers were artificially aged (80 °C, 50% relative humidity for 35 and 94 days). Historic papers were included in the study in order to compare artificially aged with naturally aged gelatin. The aqueous extracts from the papers were characterised by aqueous size-exclusion chromatography (SEC) using four PL-Aquagel-OH columns and UV photodiode array detection at 220, 254 and 280 nm. Results showed that gelatin undergoes hydrolysis upon aging, type A gelatin showing a faster degradation rate than type B. The result was an increase in the lower-molar-mass fractions, under 50 000 g mol^{-1}, and especially in a characteristic fraction with a peak molecular mass (M_p) of 14 000 g mol^{-1}. A significant decrease in the extraction yields of α-, β- and γ-chains occurred after aging. This was attributed to crosslinking, leading to the formation of less-soluble polypeptides with very high molar mass (>800 000 g mol^{-1}). Less than 10% alum had no impact on the degradation rate; higher alum contents accelerated hydrolysis reactions.

Keywords: Paper; Gelatin; Proteins

1. Introduction

Since the beginning of papermaking in Western Europe, gelatin was used to size (sizing refers to the resistance of a sheet of paper to the sorption of water) papers in order to improve the buffer effect and feathering of the inks. The size was prepared by boiling animal hides, skins and bones in water. For the finest quality papers, sturgeon gelatin could be used [1]. Initially, papermakers added aluminium salts (alum) in order to decrease paper permeability. Additionally, alum also retarded biodeterioration and decreased the viscosity of the size [2,3]. The use of alum persisted and rosin/alum was used to size mechanical woodpulp papers well into the 20th century. Nowadays, papers are sized with synthetic sizes such as alkyl ketene dimers (AKDs) and alkenyl succinic anhydrides (ASAs), which were

*Centre de Recherches sur la Conservation des Documents Graphiques, Centre National de la Recherche Scientifique, UMR 8573, 36 rue Geoffroy-Saint-Hilaire, 75005 Paris, France. Tel.: +33-1-4408-6990; fax: +33-1-4707-6295.

E-mail address: aldupont@mnhn.fr (A.-L. Dupont).

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developed for the paper industry in 1953 and 1974, respectively [4], but gelatin/alum sizing continues to be used for artist quality papers.

Collagens are the most abundant and ubiquitous proteins on earth. Most collagens are fibrillar proteins. Three left-handed α-chains form a collagen molecule, two α1 and one α2 are intertwined to form a right-handed triple helix called a γ-chain [5–8]. α1 and α2 contain about 1000 amino acids each. They have a molar mass (M_r) of 95 000 g mol^{-1} and differ slightly in the composition of the telopeptides (C and N terminal), α2 being richer in basic amino acids.

Collagen is structured in highly ordered regions (crystalline) alternating with less ordered regions (amorphous). The high proportion of amino acid triplets (glycine–X–Y) in the α-chains, where X is most often proline (Pro) and Y is most often hydroxyproline (Hyp), is responsible for the compact crystalline structure. Hyp is found exclusively in collagen. Gly (glycine), Pro and Hyp represent more than 50% of the amino acid content, Gly alone accounting for more than 30%. The amorphous regions are present mostly in the telopeptides. They form globular ends and are rich in polar amino acids with bulky side-chains, such as arginine (Arg), lysine (Lys), aspartic acid (Asp) and glutamic acid (Glu). Differences in length, charge distribution and structure of the telopeptides lead to different possible assemblies forming the quaternary structure of the protein. The latter is responsible for the different physiological functions of collagens [5,8,9].

Gelatin is produced by partial hydrolysis of collagen either in alkaline or in acid medium, both treatments resulting in a partly denatured protein. Gelatin from bovine origin is alkali produced (type B) and acid-treated gelatin (type A) is from porcine or fish origin. Depending on the origin of the protein there are some differences in the physical properties and in the amino acid content [10]. Treatment results in the individualization of a high proportion of the α-chains. Multimers of α-chains linked together, such as β-chains (α-chain dimer) and non-hydrolysed or partially renaturated triple helices, are also present in gelatin, albeit in smaller amounts. Low-molar-mass peptides (500–4000 g mol^{-1}) and polypeptides of approximately 30 000 g mol^{-1} arising from the degradation of the α-chains are formed [10–12].

Scientific research on gelatin is mainly driven towards photographic, food and pharmaceutical applications. This research provides valuable information on gelatin as a biomaterial, and on its chemical and physical properties. However, research in paper conservation has its own particular problems. A prime concern for ensuring the conservation of our cultural heritage is understanding the degradation pathways of the materials used to produce paper-based artefacts of artistic and historical value. Hydrolysis and oxidation occur during the aging process of cellulose and result in a progressive weakening of the physical strength of the paper over time. Counteracting these reactions and limiting their occurrence is crucial for improving the stability and longevity of paper artefacts. The task is far from simple, the numerous components of paper other than cellulose, whether of organic origin, e.g. starch and gelatin, or mineral and synthetic origin, e.g. optical brighteners, fillers and synthetic sizes, are numerous parameters contributing to the complexity of the chemistry taking place during natural aging. In particular, the role of the size in the aging process of paper has largely been ignored.

The work presented here shows how gelatin degrades upon aging in paper (at 80 °C, 50% relative humidity, RH) and how the presence of aluminium potassium sulphate influences the aging behaviour. This study is part of broader research into the relationships between gelatin, alum and cellulose. We analysed Whatman No. 1 filter papers sized with different combinations and concentrations of gelatin and gelatin/alum. Naturally aged historic papers as well as modern watercolour papers (Arches) were also included in the study. They were all, except for the historic papers, artificially aged at 80 °C and 50% RH for 35 and 94 days. Aqueous extracts from the papers were characterised by size-exclusion chromatography (SEC) on a set of four PL-Aquagel-OH columns (Polymer Labs.) using UV photodiode array detection (DAD).

2. Experimental

2.1. Sample description

Modern and historical (17th and 18th centuries) gelatin sized papers were selected for this study. The

modern papers included Whatman No. 1 filter paper, made of pure cellulose, and Arches (cold pressed), a 100% cotton paper from Canson. Arches paper was sized to saturation by the manufacturer with a type B gelatin. Whatman No. 1 paper was manually sized in our laboratory with gelatin type A and type B. We chose a photographic grade type B gelatin by Kind and Knox produced from alkali-treated cattle bones (further referred to as ''KK'') and a pharmaceutical/food grade type A gelatin by Norland produced from acid-treated fish skin (further referred to as ''N''). The sizing was done by immersing the Whatman No. 1 sheets (150×190 mm^2) one by one in aqueous solutions of gelatin kept at 40 °C in a thermostated water bath.

In order to achieve dry mass uptakes of gelatin in the papers of approximately 0.5, 2 and 8% (dry gelatin mass/dry paper mass), the concentrations of the aqueous gelatin solutions needed were 2.3, 8.3 and 32.3 g L^{-1} for KK and 2.1, 8.9 and 36.1 g L^{-1} for N (calculations not detailed here). These gelatin uptakes were representative of light, mid and heavy sizing [13]. The water used was Milli-Q 18.2 MΩ cm (RiOs ElIx, Millipore). The gelatin was equilibrated at 50% RH and 23 °C prior to use [14].

A set of papers was sized in solutions of KK gelatin at 8.3 g L^{-1} (2% uptake) containing various amounts of aluminium potassium sulphate [$AlK(SO_4)_2 \cdot 12H_2O$]. Three concentrations of aluminium salts were used: 0.083, 0.83 and 2.49 g L^{-1}, i.e. 1, 10 and 30% of alum (mass of alum/mass of gelatin). These were chosen in order to cover the range of alum concentrations used in historical and modern sizing practices [1,15–19].

2.2. Artificial aging

In each series, one set of papers was kept in the dark at 23 °C, 50% RH, and two sets were aged for 35 and 94 days at 80 °C, 50% RH individually (hanging sheets) in a heat/humidity aging chamber. The papers without alum were aged in a SE-600-3 Thermotron chamber (Thermotron Industries) and the papers containing alum were aged in a Versatenn chamber (Tenney Engineering). Under these aging conditions, gelatin remained under its glass transition temperature [20,21].

A few grams of KK granules and N flakes were aged in glass beakers for 35 days at 80 °C, 50% RH in the Versatenn chamber.

2.3. Conditions

2.3.1. Chemicals

Sodium dodecyl sulphate (99%), disodium hydrogenphosphate heptahydrate and sodium dihydrogenphosphate monohydrate were purchased from Acros Organics (NJ, USA). L-Alanine was from the amino acid standard kit 22 from Pierce (Rockford, IL, USA). Sodium polystyrene sulfonate (PSS) standards (M_r range 1600–1 200 000 g mol^{-1}) were from Scientific Polymer Products (Ontario, NY, USA). Gelatins used were Gelita Photographic Gelatin Type 8039, Lot 1 from Kind and Knox Gelatin (Sioux City, IA, USA) and ''High Molecular Weight Gelatin'' batch No. 7345 from Norland Products (New Brunswick, NJ, USA). Electrophoresis ''Low Molecular Weight Calibration Kit'' (Phosphorylase *b*, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and α-lactalbumin) and ''High Molecular Weight Calibration Kit'' (thyroglobulin, ferritin, catalase, lactate dehydrogenase and albumin) were purchased from Pharmacia.

2.3.2. Method and instrumentation

All analyses were carried out on a Hewlett-Packard liquid chromatograph HP 1090 equipped with a built-in thermostated column compartment. UV detection was performed using a photodiode array detector (series L Diode Array Detector). A set of four PL-Aquagel-OH columns (Polymer Labs.) 300 mm×7.5 mm and 8 μm particle diameter, 50-40-40-30, preceded by a guard column (Polymer Labs.) were connected in series. The molar mass operating range of the columns was 50 000–1 000 000 g mol^{-1} for column 50, 10 000–200 000 g mol^{-1} for columns 40 and 100–30 000 g mol^{-1} for column 30. The packing is made of a rigid macroporous material with a highly hydrophilic polyhydroxylated surface. The mobile phase was 18 g L^{-1} sodium dodecyl sulphate (SDS) in Milli-Q water and was filtered through 0.45 μm filters AH (Millipore) prior to use. SEC runs lasted 73 min at a flow-rate of 0.5 mL min^{-1} (±10 μL min^{-1}) and the sample injection volume was 20 μL. Gelatin concentration in the

samples ranged from 1 to 5 μg μL^{-1}. The temperature of the column compartment was set to 50±0.5 °C. UV detection was carried out at 220, 254 and 280 nm.

220 nm is commonly used for the detection and quantitation of peptides, the amide bond absorption range being 210–225 nm. In addition, absorptions at 254 and 280 nm were used because they are selective for tyrosine (Tyr) and phenylalanine (Phe). These two aromatic amino acids are located mainly in the telopeptidic (amorphous) regions and represent together only about 1.5% of the total amino acid content in bovine gelatin and about 2% in fish gelatin.

The use of sodium dodecyl sulphate in SEC is widespread. SDS is an anionic surfactant, complexing with the polypeptides allowing them to unfold into a rod-shaped conformation. This minimizes the error in M_r determination due to differences in the hydrodynamic volumes between PSS and gelatin and among the gelatin polypeptides themselves. Additionally, the negative charges on SDS help reduce the problem of ionic interactions between the solutes in the mobile phase and the stationary phase. However, it should be noted that the cold water extract pH [22] of all the gelatin sized papers were above 5.07, the isoelectric point (p*I*) of gelatin—with the exception of aged papers with 10 and 30% alum, which were slightly below pH 5. Under the experimental conditions, gelatin in water extracted from the papers was mostly charged negatively.

Both pH and ionic strength are known to influence the elution volume of polypeptides [23]. The choice of the mobile phase was made after comparing 1.8% SDS in water with 1.8% SDS in 50 m*M* phosphate buffer pH 6.63, in order to check if a better resolution in the chromatograms of gelatin could be obtained with a buffer of higher ionic strength and stable pH. The buffer was prepared with 4.5 g of $[NaH_2PO_4 \cdot H_2O]$ and 4.64 g of $[Na_2HPO_4 \cdot 7H_2O]$ in 1 L 1.8% SDS in water. Several gelatin samples were extracted in the mobile phase from papers without and with alum (10%), aged 35 and 94 days and unaged. The elution volume of the gelatin fractions increased: the hydrodynamic volume of gelatin in phosphate buffer was smaller than in the unbuffered, low ionic strength mobile phase. The molar mass distribution (MMD) profiles for unaged gelatin were similar to the MMD profiles in 1.8% SDS in water, only shifted slightly towards lower M_r. For the aged gelatin from papers with and without alum, the MMD profiles were markedly shifted to lower M_r and the resolution of peaks A, B and C was less than with 1.8% SDS in water. Consequently, it was decided to run SEC with 1.8% SDS in water for sample preparation and mobile phase.

2.3.3. Sample preparation

The papers were equilibrated for several days in a temperature and humidity controlled room at 23 °C, 50% RH [14]. They were cut into 3–4 mm^2 pieces. 0.5 g was weighed in a test tube and 2 mL of mobile phase was added. After 45 min incubation at room temperature, the supernatant was withdrawn and heated to 50 °C for 15 min in a thermostated water bath. The samples were filtered through poly(vinylidene difluoride) (PVDF) filters, 0.45 μm pore, 4 mm diameter (Alltech), before injection.

Gelatin solutions were also prepared by dissolving 2.5 mg of KK granules and 2.8 mg of N flakes in 1 mL mobile phase for 1 h at 50 °C (water bath), preceded by overnight soaking.

2.4. Calibration

A total exclusion volume (V_e) of 17 mL was determined by injecting a sodium polystyrene sulfonate (PSS) standard with a weight-average molar mass (M_w) of 1 188 400 g mol^{-1}. A total permeation volume (V_0) of 35 mL was determined by injecting L-alanine (89.1 g mol^{-1}). The calibration curve for M_w determination was constructed using 11 PSS standards dissolved in mobile phase at concentrations of 0.2 to 0.3 μg μL^{-1}. M_w values of PSS are 1 188 400, 801 100, 505 100, 262 600, 127 000, 57 500, 34 700, 16 600, 8000, 4950 and 1640 g mol^{-1}. The calibration curve is shown in Fig. 1. The resulting polynomial equation for M_w as a function of retention time (t_R) using a cubic fit match (1) was used for all the M_r determinations:

$$\log M_w = -6.41006 \cdot 10^{-4} t_R^3 + 8.64594 \cdot 10^{-2} t_R^2 - 4.00442 t_R + 68.51491 \quad (1)$$

Among the polymer standards tested, PSSs were

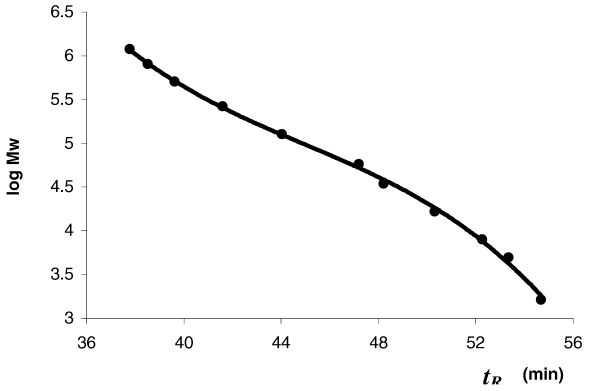

Fig. 1. Weight-average molar mass calibration with sodium polystyrene sulfonate narrow standards; $\log M_w$ as a function of retention time t_R (min).

chosen for calibration because they provided the best M_r estimation for SDS–gelatin complexes. They are soluble in water and absorb significantly at 220 nm. PSSs were prepared in mobile phase for consistency with the preparation of the samples, but it is likely that SDS does not complex with PSSs, both molecules being charged negatively. This assumption was corroborated by our experience showing that, whether dissolved in mobile phase or in water, PSSs eluted with exactly the same retention volumes. On the other hand, M_r determination of the eluted gelatin fractions using a calibration curve constructed with SDS–globular protein complexes (electrophoresis molecular mass standards from Pharmacia) resulted in significantly underestimated values (results not shown). This is due to the difference in protein families and confirms that SDS–protein complexes derived from globular proteins cannot be used as M_r standards for SDS–collagen derivatives.

2.5. Method validation (220 nm): precision, linearity and limit of detection

Calibration curves for quantitation of gelatin were constructed for KK and N. The data were also used for assessing the precision and linearity of the method and the detection limit and to estimate the extraction yields of gelatin from unaged paper samples (Section 3.1.1.1).

Table 1 shows the relative standard deviations (RSDs) in retention time (t_R), peak area, height and width obtained with eight injections performed on four different days of a mix of two polystyrene sulfonate standards of 8000 g mol^{-1} (0.285 μg μL^{-1}) and 262 600 g mol^{-1} (0.28 μg μL^{-1}). The method showed good repeatability in t_R (RSD $\leq$0.5%). Peak area, height and width have slightly higher RSD values, which is probably mostly due to the positioning of the baseline. However, it is not unusual to obtain such RSD values with aqueous SEC methods, which are extremely dependent on the precision of the instrument and on minute fluctuations in the run parameters, usually due to the precision of the delivery system. The precision of the HP 1090 solvent delivery system was ±2%. Ideal SEC conditions require a precision of 0.1% in flow-rate.

Table 1
Relative standard deviations in retention time (t_R), peak area, peak width and peak height on eight injections of a mix of PSS 8000 g mol^{-1} and PSS 262 000 g mol^{-1}

	RSD (%)			
	t_R (min)	Area (mAU min)	Height (mAU)	Width (min)
PSS 8000	0.41	2.36	2.92	2.37
PSS 262 000	0.38	3.53	3.13	4.52

The linearity of the method was assessed with solutions of gelatin (unaged) of known concentrations: a stock solution of KK at 20.71 μg μL^{-1} and six dilutions (1/2, 1/8, 1/16, 1/64, 1/128, 1/256) and a stock solution of N at 40.25 μg μL^{-1} and six dilutions (1/4, 1/8, 1/16, 1/32, 1/128, 1/256). The areas subtending the entire chromatograms were integrated and plotted versus concentration:

$$\text{Area} = 12\,864c + 475 \quad (2)$$

for KK, and

$$\text{Area} = 11\,764c + 210 \quad (3)$$

for N (where area is in mAU min and c is the concentration of gelatin in μg μL^{-1}), and were used for quantitation measurements. Correlation coefficients were 0.9999 for KK and 0.9998 for N.

In order to determine the limit of detection (LOD), signal-to-noise ratios (S/N') for the smallest peak (C) in the chromatograms at a peak molar mass (M_p) of 14 000 g mol^{-1} (Fig. 2) were calculated with the

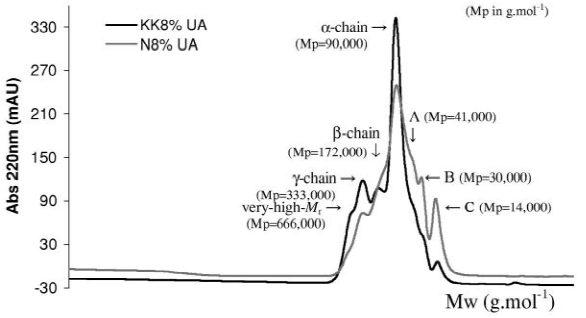

Fig. 2. Overlaid chromatograms of N gelatin and KK gelatin extracted from 8% uptake unaged Whatman No. 1 paper.

solution stock/256 (i.e. $8.5 \cdot 10^{-4}$ μg μL^{-1} of KK and $1.6 \cdot 10^{-3}$ μg μL^{-1} of N). S/N' values obtained were 30 for KK and 6 for N. The concentrations of gelatin in all the injected samples were at least 10^3 times higher.

3. Results and discussion

3.1. SEC of gelatin extracted from Whatman No. 1 papers

3.1.1. Papers without alum

3.1.1.1. Extraction yields of gelatin from unaged and aged papers

Both the unaged KK granules and granules aged 35 days dissolved completely in the mobile phase. We checked whether cross-comparisons for quantitation were possible between unaged and aged gelatin based on the respective UV absorption. The recovered mass of gelatin for aged KK granules was back-calculated by integrating the area subtending the entire chromatogram using Eq. (2). The calculated mass matched the actual known injected mass. This result confirmed that the equation established for quantitation of unaged gelatin (2) could also be used for the quantitation of aged gelatin.

Using Eqs. (2) and (3), the extraction yields of gelatin from unaged Whatman No. 1 paper were found to range from 72 to 86% for KK and from 43 to 47% for N. The theoretical concentrations were calculated based upon the dry mass uptakes, and the calculated injected masses were estimated by integrating the entire chromatogram area. For the aged Whatman No. 1, the extraction yields of gelatin were significantly lower, between 13 and 24% for KK and between 23 and 33% for N.

3.1.1.2. M_r determination and MMD profiles of gelatin from unaged papers

Fig. 2 shows MMD profiles of KK and N extracted from unaged papers with 8% gelatin uptake. The major peak had an M_p value of 90 000 g mol^{-1}. This fraction was attributed to the α-chains. The high-M_r portion of the chromatograms showed a shoulder of M_p 666 000 g mol^{-1} and two small peaks of M_p 333 000 and 172 000 g mol^{-1} for KK—respectively two shoulders and a peak for N. The latter two were attributed respectively to β-chains and γ-chains non-degraded or reformed upon cooling of the gelatin after production [5]. The very-high-M_r polypeptides (M_w 666 000 g mol^{-1}) were multimers of α-chains probably formed by aggregation or cross-linkage of α-, β- and γ-chains and are not present in native collagen [5]. These stable aggregates seem to be a particular feature of gelatin, and they were still present in the chromatogram of samples of KK diluted down to $8 \cdot 10^{-2}$ μg μL^{-1} (stock/256).

The low-M_r regions showed two shoulders with M_p values of 41 000 and 30 000 g mol^{-1} (A and B) and a small peak of M_p 14 000 g mol^{-1} (C) for KK—respectively a shoulder (A) and two peaks (B and C) for N. These low-M_r fractions resulted from the degradation of collagen during the production of gelatin [10]. Overall, it was observed that the MMD profile of N was slightly more weighted in the low-M_r regions than the MMD profile of KK. This observation corroborated results found in the literature [10].

The chromatograms of gelatin extracted from unaged papers with 2 and 0.5% uptake and the chromatograms of unaged KK granules and N flakes were similar to those shown in Fig. 2. The sizing process did not change the MMD of the gelatin to any detectable level regardless of the concentration of gelatin in solution.

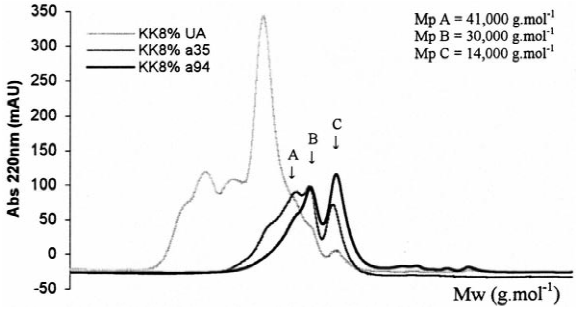

Fig. 3. Overlaid chromatograms of KK gelatin extracted from 8% uptake Whatman No. 1 paper unaged (KK8% Ua), aged 35 days (KK8% a35) and aged 94 days (KK8% a94) at 80 °C, 50% RH.

3.1.1.3. M_r determination and MMD profiles of gelatin from aged papers

Three MMD profiles of gelatin extracted from papers with 8% gelatin uptake unaged, aged 35 days and aged 94 days are shown in Fig. 3 (KK) and Fig. 4 (N). There was a significant decrease in high-M_r fractions in the extract upon aging. In the 35-day aged paper extracts, the γ-chain fraction was absent while the β-chain fraction was barely present and reduced to a small tailing. The α-chain fraction was considerably reduced. After 94 days, the β-chain tail disappeared and the α-chain fraction was further reduced. The decrease in high-M_r fractions was likely due to a decrease in the solubility of this polypeptidic portion as observed previously (Section 3.1.1.1). This could arise from cross-linking of the high-M_r fractions of the gelatin during aging or from a binding of the polypeptides to the cellulose molecules through links other than hydrogen bonds. Fourier transform infrared spectroscopy using an attenuated total reflectance probe (Nicolet Avatar 360 FTIR) was performed on the papers after extraction in order to verify whether residual gelatin was left, but the result proved inconclusive since no protein bands were clearly evidenced.

The main peaks in the chromatograms of the extracts of aged papers were the three low-M_r fractions corresponding to peaks A, B and C (Figs. 3 and 4). Upon aging, peak C increased considerably while peak B decreased and peak A progressively disappeared. Hydrolysis of high-M_r γ-, β- and α-chains seemed to occur at preferential weak points, leading to this substantial increase in specific low-M_r fractions. This result confirmed the findings of a study on the degradation of photographic gelatin induced by pollution. Exposure to a mixture of SO_2 (27 mg m^{-3}) and NO_2 and (38 mg m^{-3}) for 18 and 30 days resulted in fewer high-M_r fractions, while a characteristic low-M_r fraction below 20 000 g mol^{-1} was shown to increase [24].

Peak C, the main characteristic fraction formed in both 35- and 94-day aged samples, could be an indicator of gelatin degradation upon aging. A plot of peak C height versus aging time showed a non-linear but steady increase (Fig. 5). A kinetics study would be necessary to gain more insight.

A hypothesis for preferential hydrolysis points

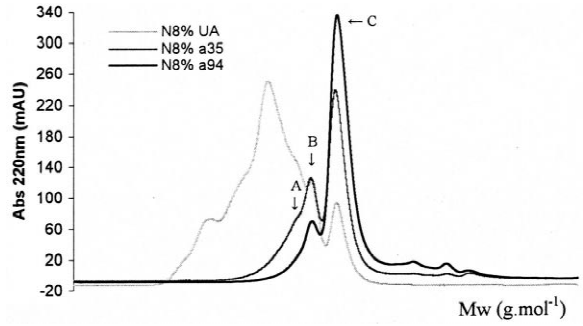

Fig. 4. Overlaid chromatograms of N gelatin from 8% uptake Whatman No. 1 paper unaged (N8% Ua), aged 35 days (N8% a35) and aged 94 days (N8% a94).

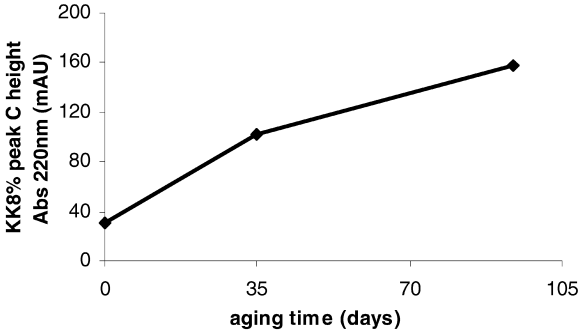

Fig. 5. Peak C height in mAU, detection at 220 nm (KK gelatin extracted from 8% uptake Whatman No. 1 paper) as a function of aging time in days (values averaged from two different samples).

leading to specific low-M_r fractions can be proposed. It is known that amino acids in peptides are unequally sensitive to partial hydrolysis [25]. For instance, in a dipeptide the positively charged ammonium group close to the amide bond tends to repel acidic protons. The amide bond of a dipeptide is thereby more stable than an analogous bond in a polypeptide. On the opposite end, aspartyl residues are very susceptible to hydrolysis in dilute acid, because the negatively charged carboxyl groups of aspartic acid attract hydrogen ions, which decreases the stability of the neighbouring peptide bonds. Other effects, such as steric hindrance, are involved in the relatively better resistance to acid hydrolysis of peptides with valine and leucine as amino-terminal residues. In this case, the isopropyl and isobutyl side chains of valine and leucine hinder the approach of the acidic proton. These effects are more complex and less known for polypeptides. In the case of the degradation of gelatin upon heat/humid aging, partial-hydrolysis specificity is quite likely to be involved and responsible for the specific low-M_r fragments formed.

Fig. 6 shows three MMD profiles: unaged KK granules, KK granules aged 35 days and KK extracted from 2% uptake papers aged 35 days. A new peak with M_p 830 000 g mol^{-1} appeared for the aged KK granules. There were also fewer γ-, β- and α-chains, but higher proportions of low-M_r fractions (A, B and C) in the aged KK granules as compared with unaged KK granules. Hence, the aging of KK granules resulted in two distinct outcomes taking place concomitantly. On the one hand, there was a substantial increase in the low-M_r fractions, especially in the peak C fraction. On the other hand, very-high-M_r polypeptides (M_p >800 000 g mol^{-1}) were formed. The latter most likely arose from crosslinking or aggregation of some of the β- and γ-chains [26]. This presence of crosslinked networks in high-M_r gelatins as a structural feature retained from the native collagen structure has been observed by other authors [27].

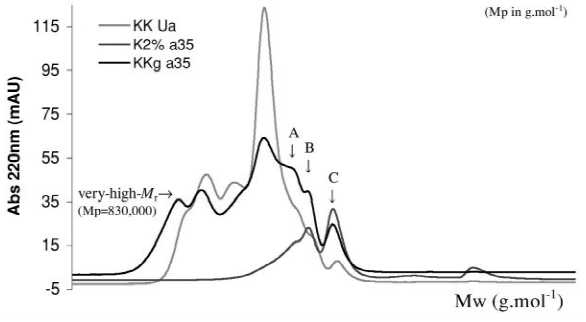

Fig. 6. Overlaid chromatograms of KK gelatin granules unaged (2.59 μg/μL) (KKg Ua), KK gelatin granules aged 35 days (2.5 μg/μL) (KKg a35) and KK gelatin extracted from 2% uptake Whatman No. 1 papers aged 35 days (estimated 4.7 μg/μL) (K2% a35).

The presence of crosslinked fractions was consistent with the low mass recovery and with the observation made throughout this study of the absence of high-M_r fractions in the gelatin extracts from aged papers. Crosslinking arising from a progressive dehydration of the bound water of gelatin seemed to occur in sized paper upon aging and resulted in a solubility decrease.

However, we observed in the case of KK granules that, after aging, the crosslinked fraction was still soluble in the mobile phase. One hypothesis to explain why, when gelatin was aged in paper, the crosslinked fraction became insoluble, involves the role of sugars and aldehydic compounds and their favouring of protein crosslinking. Numerous sugars and oxidised sugars have been identified as degradation products of cellulose upon aging [28,29] and recent research showed that they decrease the solubility of gelatin by promoting crosslinking of the protein [30]. The proposed mechanism is via an Amadori rearrangement [31] where the aldehyde group of a reducing sugar can react with a free amino group of gelatin, resulting in the formation of an amino glycoside, which can further react with another gelatin amino group, thereby giving rise to the crosslinked structure. Because of their dialdehydic nature, sugars oxidised with periodic acid, for instance, were shown to be more efficient and lead to a more complete interaction with gelatin polypeptides [30]. Bonding between the gelatin and the cellulose molecules was probably also involved to some extent, since oxidised radicals on the cellulose can form without cleavage from the polysaccharidic chain. Polysaccharide–protein interactions are complex. Earlier work showed that hydrogen bonds between non-substituted hydroxyl groups of methylcellulose and carboxyl groups of gelatin

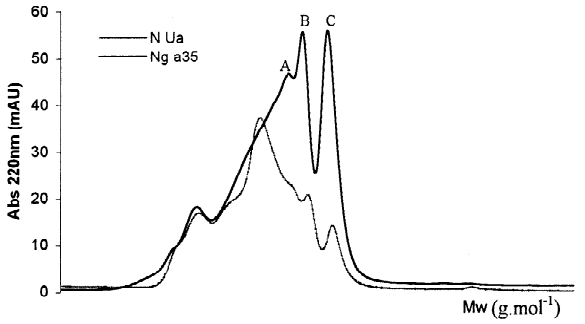

Fig. 7. Overlaid chromatograms of N gelatin granules unaged (1.25 μg/μL) (Ng Ua) and N gelatin flakes aged 35 days (estimated 2.1 μg/μL) (Ng a35).

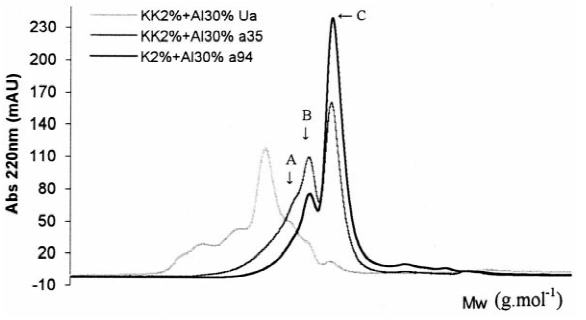

Fig. 8. Overlaid chromatograms of gelatin extracted from Whatman No. 1 papers sized with KK 2% uptake and 30% alum unaged (KK2% + Al30% Ua), aged 35 days (KK2% + Al30% a35) and aged 94 days (KK2% + Al30% a94).

form when both components are mixed in certain proportions [32].

Fig. 7 shows MMD profiles of unaged N flakes and N flakes aged 35 days. As calculated using Eq. (3), only an estimated 26% of the latter dissolved in the mobile phase. The very-high-M_r fraction (M_p $>800\,000$ g mol^{-1}) was only a small shoulder in both chromatograms. This result suggested that, in the 35-day aged N flakes, only a small part of the very-high-M_r fraction was soluble. Here also, N showed a faster degradation rate than KK.

3.1.2. Papers with alum

The MMD profiles of the gelatin from papers containing 1 and 10% alum (mass alum/mass gelatin) were similar to those with no alum for each respective aging time (not shown). The hydrolysis of gelatin due to aging was not accelerated by the presence of alum in quantities of up to 10%. However, 30% alum in the size resulted in a more extensive hydrolysis of the gelatin to smaller peptides upon aging. After 35 days aging, the MMD profile was similar to that of 94-day aged samples containing no alum. After 94 days, profiles showed a significantly decreased peak B and an increased peak C (Fig. 8).

It was also observed that the extraction yields of gelatin from unaged papers decreased with an increase in alum content (not shown). The role of alum as a hardener of gelatin is well known, especially in photography. Aluminium salts react with the ionised carboxyl groups of gelatin [12,33,34]. It can be hypothesized that the mechanism by which alum formed bridges between gelatin and cellulose was most likely similar to the mechanism described in the literature by which alum forms bridges between rosin and cellulose in rosin/alum sizing (Fig. 9) [35]. Results showed that while the areas subtending the chromatograms of the gelatin extracted from the aged papers without alum and the aged papers with 1% alum were similar, the areas subtending the chromatograms of gelatin from aged papers with 10 and 30% alum increased proportionally to the increase in alum content. In the aged papers, the height of the low-M_r peaks increased with the alum content.

Upon aging, 10 to 30% alum accelerated the

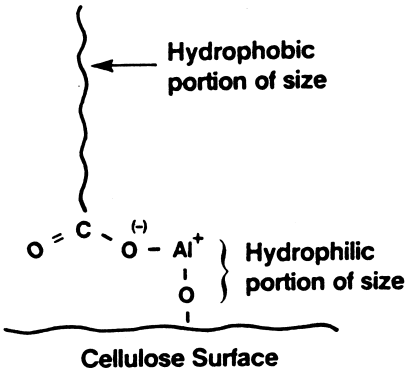

Fig. 9. Model of aluminium bridge between cellulose surface and a size [4].

hydrolysis of gelatin, producing a higher proportion of easily extractable low-M_r fractions. Thirty percent alum additionally resulted in a more extensive hydrolysis of the protein to smaller peptides.

3.2. SEC of gelatin extracted from Arches papers

Fig. 10 shows chromatograms of unaged and aged Arches papers. The high-M_r fraction was absent in the chromatogram of the unaged Arches paper. The further shift towards low-M_r fractions upon aging occurred earlier than for the Whatman No. 1 papers. Gelatin extracted from unaged Arches paper was poor in high-M_r molecules, γ-, β- and α-chain fractions were small shoulders and the MMD profile was similar to the profile of gelatin from the 8% KK uptake Whatman No. 1 papers aged 35 days. The Arches papers were kept in the laboratory for 10 years before analysis. Despite the fact that the commercial sizing procedure and the grade of the gelatin used by the manufacturer could not be documented, the result suggested that, under natural aging conditions, the two phenomena observed—hydrolysis and crosslinking—started fairly early in time. Analysis of the Arches papers with scanning electron microscopy/energy dispersive X-ray (SEM/EDX) (JEOL JSM 5410 LV SEM/Oxford EDS system) showed the presence of aluminium. The presence of alum in the Arches papers could explain the high degradation rate of the gelatin.

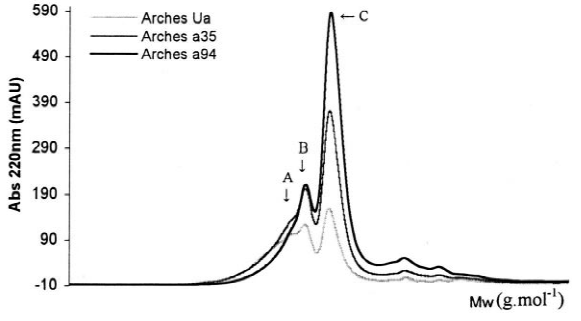

Fig. 10. Overlaid chromatograms of gelatin extracted from Arches (Canson) papers unaged (Arches Ua), aged 35 days (Arches a35) and aged 94 days (Arches a94).

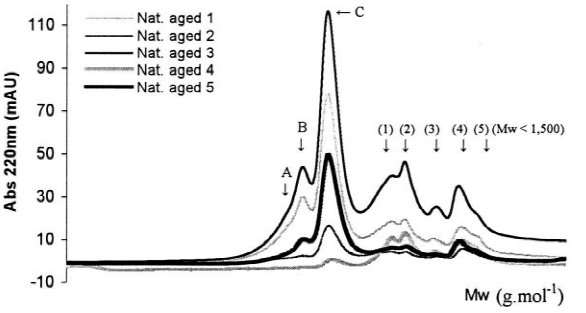

Fig. 11. Overlaid chromatograms of gelatin extracted from five different naturally aged papers (17th and 18th centuries).

3.3. SEC of gelatin extracted from naturally aged papers

Fig. 11 shows the chromatograms of five different naturally aged historic papers sized with gelatin (17th and 18th centuries). The MMD profiles were skewed towards the low-M_r region, showing peaks B and C characteristic of highly degraded gelatin. The closest match for the MMD profiles were the profiles of gelatin extracted from papers sized with KK aged 94 days, but peak A (shoulder) was even smaller.

Across the very-low-M_r end in the chromatograms of all naturally aged papers, a series of five small peaks were found spreading from t_R 56 to 62 min ($M_p < 1500$ g mol^{-1}). The very last of these small peaks (5) (Fig. 11) was most likely a ghost peak since it was also present in the chromatograms of unaged gelatin granules and in the chromatograms of extracts of control Whatman No. 1 unaged unsized (not shown). The three small peaks (1, 2 and 4) were exclusively found elsewhere in the chromatograms of the extracts of aged Whatman No. 1 papers (sized and unsized) and Arches papers. No correlation could be found between the area of these peaks and the amount of gelatin and/or alum in the papers. These three small peaks were attributed to soluble UV-absorbing degradation products from cellulose and in that respect could be indicative of extensive degradation of the paper [28]. The very small peak (3) seemed to be present only in the chromatograms of aged, sized papers and Arches papers and probably arose from the aging of gelatin.

3.4. Absorption at 254 and 280 nm

Detection at 254 and 280 nm provided useful additional information. Absorption at either wavelength was very low (2 to 10 mAU) since Tyr and Phe are present in minute quantities in gelatin, but the general MMD profiles at 254 and 280 nm of unaged and aged gelatins resembled the profiles at 220 nm (Fig. 12). It was interesting to note that, for the major peaks ($M_w \geq 14\,000$ g mol^{-1}), the A_{220}/A_{254} ratios were single digit numbers, while for the very-low-M_r peaks (1 to 4), the A_{220}/A_{254} ratios exceeded a value of 10 or even 100. This seemed to indicate that Tyr and Phe were quite evenly distributed among the polypeptide fractions before and after aging, but that they were more numerous in the fractions of $M_w < 1500$ g mol^{-1} arising from aging. The amorphous regions of gelatin, the telopeptides, which contain a higher proportion of these amino acids, undergo, upon aging, extensive cleavage to form small peptides. The exposure of gelatin to atmospheric pollutants showed a similar degradation behaviour, where amino acids from the telopeptides were found to elute in the total-permeation peak [24].

However, as suggested earlier, chemical species other than amino acids could also be involved in the UV absorption at 254 and 280 nm of the very-low-M_r fractions—most likely, UV-absorbing species from the degradation of cellulose [28,36].

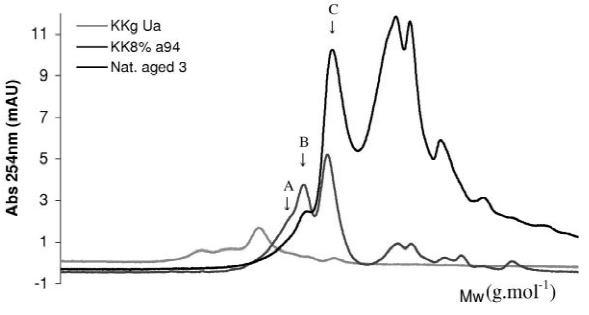

Fig. 12. Overlaid chromatograms of KK granules unaged (2.59 μg/μL) (KKg Ua), KK gelatin extracted from 8% uptake Whatman No. 1 paper aged 94 days (KK8% a94) and gelatin extracted from naturally aged paper (Nat. aged 3). Detection at 254 nm.

4. Conclusions

Upon heat/humid aging the gelatin in paper underwent significant degradation. The α-chains were shown to break at weak points, producing specific low-M_r fractions. The peptide bonds were cleaved and native γ-, β- and α-chains were progressively lost, concomitantly giving rise to two main low-M_r fractions. A fraction with M_p 14 000 g mol^{-1} (peak C) was the most characteristic fraction to increase significantly upon aging. Peak C could potentially be used as a marker for monitoring gelatin degradation. Peak C was also present in very small quantities in unaged gelatin, forming the so-called ''non-gel'' portion [9,10], i.e. small fragments produced by cleavage of the α-chains during the production of gelatin. A hypothesis for preferential hydrolysis points involving the different sensitivities of the amino acids in peptides to partial hydrolysis was proposed. The characterization of the terminal amino acids of the peptides in the low-M_r fractions using hyphenated mass spectrometry techniques would help clarify this phenomenon and possibly determine the most labile amide bonds in gelatin.

Another remarkable observation in this study was the formation upon aging of a very-high-M_r fraction of $M_p > 800\,000$ g mol^{-1}, i.e. well above the M_r of native collagen γ-chains, which appeared at the same time as hydrolysis proceeded in other areas of the protein to yield low-M_r fractions. This very-high-M_r fraction, which cannot be extracted from aged sized papers, most likely arose from crosslinking between the γ-, β- and α-chains. The crosslinking was a consequence of the heat/humid aging treatment and could be further enhanced by the presence of sugars and oxidised sugars produced by the degradation of cellulose.

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References

[1] J.J. Diderot, Encyclopédie ou Dictionnaire Raisonné de Sciences, des Arts et des Métiers, par une Société de Gens de Lettres, A. Neufchastel, Samuel Faulche et Compagnie, Vol. 11, 1765.
[2] I. Brückle, Abbey Newsl. 17 (4) (1993) 53.
[3] I. Brückle, in: S. Fairbrass (Ed.), Conference Papers Manchester 1992, Institute of Paper Conservation, Leigh Lodge, 1992, p. 201.
[4] J.C. Roberts, in: J.C. Roberts (Ed.), Paper Chemistry, 2nd ed., Blackie, Glasgow, 1996, p. 140, Chapter 9.
[5] W. Traub, K.A. Piez, in: C.B. AnfisenJr., J.T. Edsall, F.M. Richards (Eds.), Advances in Protein Chemistry, Vol. 25, Academic Press, New York, 1971, p. 243.
[6] A. Rich, F.H.C. Crick, J. Mol. Biol. 3 (1961) 483.
[7] G.N. Ramanchandran (Ed.), Treatise on Collagen, Vol. 1, Academic Press, New York, 1967, p. 103.
[8] J. Bella, M. Eaton, B. Brodsky, H.M. Berman, Science 266 (1994) 266.
[9] E.A. MacGregor, C.T. Greenwood (Eds.), Polymers in Nature, Wiley, Chichester, 1980, p. 142.
[10] P.I. Rose (Ed.), The Theory of the Photographic Process, 4th ed., Mcmillan, New York, 1977, p. 51, Chapter 2.
[11] J.J. Harding, in: C.B. AnfisenJr., J.T. Edsall, F.M. Richards, M.L. Anson (Eds.), Advances in Protein Chemistry, Vol. 20, Academic Press, New York, 1965, p. 109.
[12] R.J. Croome, F.G. Clegg (Eds.), Photographic Gelatin, Focal Press, London, 1965, p. 49.
[13] T. Barrett, C. Mosier, J. Am. Inst. Conserv. 34 (1995) 173.
[14] T 412 om-94, TAPPI Test Methods, Technical Association of the Pulp and Paper Industry, Atlanta, 1994.
[15] J.J.F. De Lalande, L'art de Faire le Papier, 1761.
[16] S. Green, in: S. Fairbrass (Ed.), Conference Papers Manchester 1992, Institute of Paper Conservation, Leigh Lodge, 1992, p. 197.
[17] T. Balston (Ed.), James Whatman, Father and Son, Methusen, London, 1957, p. 59.
[18] F.H. Norris (Ed.), Paper and Paper Making, Oxford University Press, Oxford, 1952, p. 228.
[19] W.H. Sanburn, personal folder notebook, Waroronoro Paper, MA, 1909.
[20] M.H. McCormick-Goodhart, J. Imaging Sci. Technol. 39 (1995) 157.
[21] P.Z. Adelstein, J.-L. Bigourdan, J.M. Reilly, J. Am. Inst. Conserv. 36 (1997) 193.
[22] T 509 om-88, TAPPI Test Methods, Technical Association of the Pulp and Paper Industry, Atlanta, 1988.
[23] S. Shinagawa, K. Kameyama, T. Takagi, Biochim. Biophys. Acta 1161 (1993) 79.
[24] T.-P. Nguyen, Thèse Doctorale, Université de Rouen, U.F.R de Sciences, 1998.
[25] R. Hill, in: C.B. Anfinsen, M.L. Anson, J.T. Edsall, F.M. Richards (Eds.), Advances in Protein Chemistry, Vol. 20, Academic Press, New York, 1965, p. 39.
[26] A.J. Bailey, S.P. Robins, G. Balian, Nature 251 (1974) 105.
[27] A. Veis, J. Cohen, Nature 186 (1960) 720.
[28] A.-L. Dupont, Restaurator 17 (1996) 145.
[29] D. Erhardt, M. Mecklenburg, C. Tumosa, in: ICOM-CC Preprints II, 11th Triennial Meeting, Edinburgh, 1996, p. 903.
[30] R. Cortesi, C. Nastruzzi, S.S. Davis, Biomaterials 19 (1998) 1641.
[31] J.E. Hodge, J. Agric. Food Chem. 1 (1953) 928.
[32] K. Nishinari, K.E. Hofmann, K. Kohyama, H. Moritaka, N. Nishinari, M. Watase, Biorheology 30 (1993) 243.
[33] D.M. Burness, J. Pouradier (Eds.), The Theory of the Photographic Process, 4th ed., Mcmillan, New York, 1977, p. 77.
[34] E.M. Brown, R.L. Dudley, A.R. Elsetinow, J. Am. Leather Chem. Assoc. 92 (1997) 225.
[35] J.M. Gess, in: J.C. Roberts (Ed.), Paper Chemistry, 2nd ed., Blackie, Glasgow, 1996, p. 128, Chapter 8.
[36] E. Eusman, Conservation Research, National Gallery of Art, Washington, DC, 1995, p. 11.