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Study of gelatin renaturation in aqueous solution by AFlFFF–MALS: Influence of a thermal pre-treatment applied on gelatin

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ABSTRACT

Asymmetrical Flow Field-Flow Fractionation coupled to Multi-Angle Light Scattering has been applied to study and understand the renaturation phenomenon of gelatin in dilute aqueous solutions. Renaturation behaviour has been studied on a native gelatin powder and compared to the same gelatin, pre-heated at 75 °C. An increase of molar mass, due to gelatin chains association at room temperature was observed for native gelatin. AFlFFF–MALS analysis allowed to follow α, β, γ chains association and the occurrence of other fractions with high molar mass. This process was absent in pre-heated gelatin which suggested that pre-heating process could lead to interactions between gelatin chains which limited renaturation process in aqueous solutions. For pre-heated gelatin samples, the average initial molar mass was higher and hardly changed during incubation of a dilute solution.

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1. Introduction

Gelatin is obtained by thermal denaturation, physical and chemical degradation of fibrous collagen from bovine hide, bone or pigskin (Ledward, 1986; Ward & Courts, 1977). Thus, in aqueous solutions, gelatin is a mixture of different polypeptide chains resulting from collagen denaturation. Three main chain sizes (α, β, γ) are generally identified in gelatin solutions; α chain is a monomer whereas β and γ correspond roughly to dimers and trimers of the α chain. The molar mass of purified α chains is close to 90×10^3 g mol^{-1}, whereas that of β and γ components can reach 180 and 300×10^3 g mol^{-1} respectively (Boedtker & Doty, 1956; Piez, 1967).

In aqueous solution at room temperature gelatin chains undergo a conformational coil to helix transition which is responsible for the gel network structure creation, if the concentration is high enough (Pezron, Djabourov, & Leblond, 1991; Veis, 1964). Self-association of gelatin chains by triple helixes formation results in an increase of apparent molar mass (Lundin et al., 2000). This has been underlined by Olivares, Peirotti, and Deiber (2006) who have shown an increase of gelatin viscosity during renaturation process, due to association phenomenon. This phenomenon produces gelatin chains associations with a wide range of molar mass. Clusters are formed through chains association involving weak bonds (i.e.: hydrogen…) between triple helixes.

Many techniques (light scattering, gel permeation, size exclusion, osmotic pressure, viscosity) have been used to determine the different fractions of gelatin in solution (Meyer & Morgenstern, 2003). Size exclusion chromatography (SEC) coupled to MALS highlights the presence of α, β and γ chains forming gelatin but also the existence of particles of hMW which could not be determined since ultra-high molecular weight (ultra-hMW) components are eluted close to the exclusion limit of the SEC columns (Viebke & Williams, 2000). Due to the lack of such drawbacks, AFlFFF is a separation technique that can be considered as an alternative (no exclusion peak, low shear stress) to SEC for the characterization of polymers, biopolymers, macromolecules, colloids (Giddings, Yang, & Myers, 1977; Wahlund, Gustavsson, MacRitchie, Nylander, & Wannerberger, 1996) and gelatin (Fraunhofer, Winter, & Coester, 2004).

We have shown in a recent work (Rbii, Violleau, Guedj, & Surel, 2009) the efficiency of AFlFFF–Mals to highlight the occurrence of very hMW aggregates leading to gelatin insolubility after a thermal pre-treatment at 75 °C applied on gelatin powder. In the present paper, aims of our works were to study the effects of the same thermal pre-treatment on renaturation phenomenon. We studied the renaturation behaviour of a standard gelatin and compared it to the renaturation behaviour of the same gelatin powder pre-heated at 75 °C.

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2. Material and methods

2.1. Native Gelatin (NG) sample

Gelatin used was kindly provided by Rousselot S.A.S (Isle sur la Sorgue, France). It was an acid pigskin gelatin with the following physical properties: Bloom: 263 g; Viscosity at 6.67%: 4.62 mPa s; Isoelectric point: 9.3; Moisture (w/w); 11%. Molar mass distribution characteristics were: $M_n = 98.5\times10^3$ g mol^{-1} and $M_w = 100.7\times10^4$ g mol^{-1}.

2.2. Thermal Pre-treated Gelatin powder (TPG) preparation

Six grams of native gelatin powder (NG) were left in 50 mL glass bottles. Bottles were closed hermetically and left in an oven at 75 °C for 2 days. Each experiment was repeated twice.

These thermal treatments lead to modification of gelatin behaviour in water as described elsewhere (Marks, Tourtellotte, & Andux, 1968; Rbii et al., 2009).

2.3. Sample preparation and incubation

NG and TPG samples were prepared as follows. First, 2 g of gelatin powder was subjected to a hydration step with 100 mL of de-ionised water (Milli-Q, Millipore, Bedford, MA, USA) (resistivity of 18.2 MΩcm) for 1 h, in order to let gelatin powder swell. Then the mixture was heated in a water bath at 55 °C until it was completely solubilised.

A set of 6 NG samples and 6 TPG samples at 2 mg/mL were prepared as follows: 1 mL of the previous solution was added to 9 mL of eluent used during AFlFFF–MALS analysis (2 mM sodium phosphate with 14 mM sodium chloride buffer pH 6.0). Samples were incubated at room temperature for 3, 5, 7, 9, 11, or 13 h and submitted to AFlFFF–MALS analysis. Experiments have been duplicated.

2.4. Fractionation and light scattering

Experimental set-up and procedures for AFlFFF were composed of an Eclipse 2 System (Wyatt Technology Europe, Dernbach, Germany), connected to a DAWN-DSP laser Photometer (Wyatt Technology, Santa Barbara, CA, US), which is a Multi-angle Light Scattering (MALS) detector with a laser wavelength $\lambda = 633$ nm and a UV detector Agilent 1100. An Agilent 1100 Series Isocratic Pump (Agilent Technologies, Waldbronn, Germany) with an in-line vacuum degasser and an Agilent 1100 Autosampler delivered the carrier flow and handled sample injection into the AFlFFF channel. A 0.1 μm in-line filter (VVLP, Millipore, Germany) was installed between the pump and the AFlFFF channel. AFlFFF channel used a 250 μm thick Mylar spacer with a trapezoidal shape. The accumulation wall was an ultrafiltration membrane of regenerated cellulose with 5 kDa cut-off (Wyatt Technology Europe, Dernbach, Germany). In all calculations, a 0.164 mL g^{-1} refractive index increment was used. For concentration detection, the absorbance was measured at 214 nm and the UV extinction coefficient value was fixed at 1.27×10^4 mL g^{-1} cm^{-1}.

Eluent used for AFlFFF–MALS analysis was a 2 mM sodium phosphate with 14 mM sodium chloride buffer, pH adjusted at 6.0 by addition of phosphoric acid (85.1% v/v). The eluent was filtered before use (vacuum filtration system using Gelman filters of 0.1 μm).

AFlFFF fractionation procedure consisted in three steps: injection, focus, and elution. For separation, the channel flow rate was fixed at 1 ml min^{-1} and the cross-flow rate varied. During injection, eluent entered into the channel by inlet and outlet and was completely wasted by the cross-flow outlet. A first step consisted of fixing the cross-flow rate at 1.5 ml min^{-1}. The flows and system pressure was then allowed 1 min to stabilize before sample injection into the channel. The sample volume injected was 30 μl at a flow rate of 0.2 ml min^{-1}. After injection, 1 min of focus was kept before the elution started. In elution mode, the sample components were then eluted at 1.5 ml min^{-1} for 6 min, and then the cross-flow rate decreased linearly during 7 min, followed by an elution at 0.2 ml min^{-1} for 40 min. Flow rate through the detectors, V_{out}, was constantly maintained at 1 ml min^{-1}.

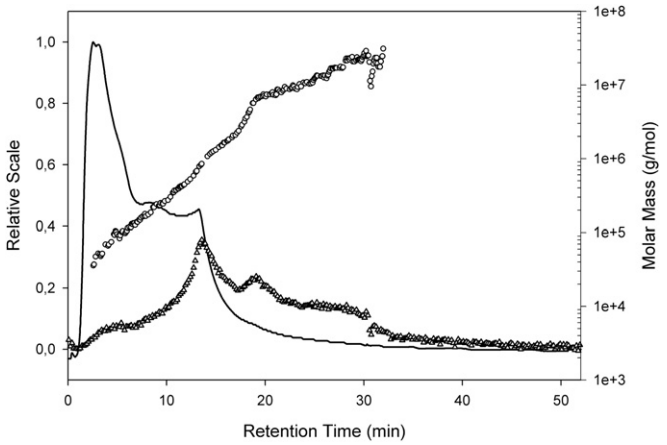

Fig. 1. Fractogramm from AFlFFF analysis of native gelatin (NG) sample at 3 h incubation time. UV signal (solid line–left axis), 90° light scattering signal (triangle up–left axis) and calculated molar mass (open circle–right axis).

Calculations of molecular weight number-average (M_n), weight-average (M_w) and mass fraction were performed using ASTRA software (version 5.3.4.14, Wyatt technology, Santa Barbara, US).

3. Results and discussion

Fig. 1 illustrates a typical fractogramm of a gelatin sample by AFlFFF–MALS at ambient temperature, with typical UV, light scattering (at 90°) and molar mass profiles. The molar mass of gelatin samples ranged from 2×10^4 to 3×10^7 g mol^{-1} which was in agreement with results obtained by Fraunhofer et al. (2004). This data confirmed the molar mass heterogeneity in the sample.

If the fractogramm was divided into five parts, it was observable from 1 to 5 min a large UV response with a low MALS signal which is specific of small molecules, that can be assigned to the α chains (monomers) with molar mass included between 5×10^4 and

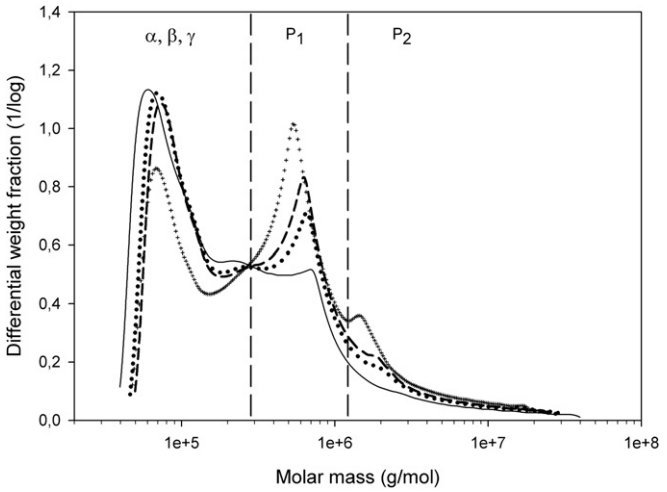

Fig. 2. Differential weight fraction vs molar mass of NG sample during incubation: 3 h (solid line); 7 h (dotted line); 9 h (small dash line); 13 h (cross line).

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