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Analysis of aged gelatin by AFlFFF-MALS: Identification of high molar mass components and their influence on solubility

Khalid Rbii [a,b], Frédéric Violleau [a,*], Sylviane Guedj [b], Olivier Surel [a]

[a] Université de Toulouse, Ecole d'Ingénieurs de Purpan, Laboratoire d'Agro-Physiologie, UPSP/DGER 115, 75, voie du TOEC, BP 57611, F-31076 Toulouse Cedex 03, France
[b] Rousselot Isle sur la Sorgue SAS, F-84800 L'Isle sur la Sorgue, France

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ABSTRACT

Gelatin is an important product for several industries and its solubility dramatically influences its functional properties. In order to be able to predict the gelatin behaviour, a new technique for its analysis has been developed with an Asymmetrical Flow Field-Flow Fractionation coupled to a multi-angle light scattering. The AFlFFF-MALS analysis showed the gelatin molar mass ranging from 5×10^4 to 2×10^7 g mol^{-1}. This technique also permitted to follow aggregation of gelatin samples after process in an oven at 75 °C. Between 0 and 4 days, some huge aggregates appeared. Their size and density increased without changing gelatin solubility. From 8 to 30 days, the molar mass and density of these aggregates increased leading to partial gelatin insolubilisation in water. This phenomenon is supposed to be due to cross-linking of the gelatin macromolecules.

1. Introduction

Gelatin is a natural biopolymer obtained by denaturation and partial hydrolysis of fibrous collagen from bovine hide, bone and pigskin. Due to its unique functional properties (gel formation, thickening effect, film formation…), gelatin is a main ingredient in food, photographic, cosmetic and pharmaceutical industries (Schrieber, & Gareis, 2007; Segtnan, Kvaal, Rukke, Schuller, & Isaksson, 2003).

In this latter field, gelatin is used in the manufacture of hard or soft capsules (Chiwele, Jones, & Podczeck, 2000; Marchais, Cayzeele, Legendre, Skiba, & Arnaud, 2003). These capsules are sometimes stored for a very long time before being used. Studies have shown that, depending on the quality of gelatin and conditions of storage (high humidity and/or high temperature), gelatin can become partially insoluble into water (Chafetz, Hong, Tsilifonis, Taylor, & Philip, 1984; Murty, Enders, & Fawzi, 1989). This causes the formation of translucent films around gelatin capsules that restrict drug release (Digenis, Gold, & Shah, 1994). As suggested by Marks, Tourtellotte, and Andux (1968)," insolubility can be due to polymerization and aggregation of gelatin molecules probably involving cross-linking and hydrogen bonding." This phenomenon could lead to the formation of high molar mass components. Consequently, the determination of the molar mass distribution could be very relevant to understand the insolubilisation phenomena.

Many techniques can be used to determine molar mass distribution of biopolymers (gel permeation, size exclusion, osmotic pressure, viscosity…) (Denis et al., 2008; Hwang et al., 1998). For gelatin analysis, molar mass distribution is usually determined by size exclusion chromatography coupled to a suitable detector (Meyer & Morgenstern, 2003). However, this method presents some restrictions like columns calibration, protein adsorption on chromatographic phase and total exclusion of very high molar mass compounds. Furthermore, it has also been reported that the disassociation of high molar mass components i.e. vesicles, micelles or weak aggregates can occur, due to the high shear forces generated in the column during the elution (Viebke & Williams, 2000).

For gelatin analysis, as described by Fraunhofer, Winter, and Coester (2004), huge aggregates preservation and characterization can be obtained using Asymmetrical Flow Field-Flow Fractionation (AFlFFF) coupled to Multi-Angle Light Scattering (MALS). This technique leads to the fractionation of proteins ranging from 1×10^4 to 1×10^7 g/mol whereas Size Exclusion Chromatography (SEC) gives size up to only 1×10^6 g/mol for the same sample. AFlFFF also fractionates ultra high molar mass components which are eluted in the exclusion peak with SEC. This kind of high molar mass aggregates are supposed to be responsible for insolubility problems with gelatin capsules. Thus, AFlFFF-MALS could be a relevant tool to investigate these phenomena.

The aim of this study was to demonstrate the ability of AFlFFF-MALS (i) to identify and characterize aggregates with high molar masses during gelatin ageing in an oven at 75 °C for 30 days (ii) to study influence of these aggregates on gelatin solubility in water.

* Corresponding author. Tel: +33 5 61 15 29 78; fax: +33 5 61 15 30 60.
E-mail address: frederic.violleau@purpan.fr (F. Violleau).

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2. Theory

2.1. Asymmetrical Flow Field-Flow Fractionation

Described for the first time in 1960 by J.C. Giddings, Flow Field-Flow Fractionation (FlFFF) is a new way of fractionation of ultra-large material from 1 kDa to a few micrometers (Myers, 1997; Wahlund, 2000). This technique is characterized by the particularity of a "soft" separation mechanism, which is ideally suitable to maintain the native structure of intact proteins (Reschiglian, Zattoni, Roda, & Cinque, 2005). Compared to other FFF methods (Sedimentation FFF – Hanselmann, Burchard, Ehrat, & Widmer, 1996 – and Thermal FFF – Lou, Myers, & Giddings, 1994), AFlFFF is more universal and efficient with a broader application range. It has been used for separation and characterization of many type of molecules of ultrahigh molar mass like biopolymers, colloids and particles (Giddings, Yang, & Myers, 1977; Litzén, Walter, Krischollek, & Walhund, 1993; Wahlund, Gustavsson, MacRitchie, Nylander, & Wannerberger, 1996). The main component in an AFlFFF system is the separation channel (Fig. 1), in which the sample is carried with an aqueous or organic eluent in a laminar parabolic flow profile. In the case of the AFlFFF, an another flow perpendicular to the carrier flow called "cross-flow" is used to generate the force field, to separate the macromolecules in function of the diffusion coefficient during elution (Fig. 2).

As separation is governed by the diffusion coefficient D, a fundamental relationship between the retention time and the diffusion coefficient of a sample can be predicted by the Eq. 1:

$$t_R = \frac{t^0 \cdot Vc \cdot w^2}{6 \cdot V^0 \cdot D} \tag{1}$$

where t_R is the retention time of the component, t^0 is the void time (that is the retention time of unretained solute), w is the channel thickness, D is the diffusion coefficient, Vc is the volumetric cross-flow rate, V^0 is the volume of the separation channel

$$D = \frac{k \cdot T}{6 \cdot \pi \cdot \eta \cdot r_H}. \tag{2}$$

If the Stokes–Einstein relationship Eq. 2 is combined with Eq. 1, the hydrodynamic diameter (d_H) can be predicted from Eq. 3.

$$d_H = \frac{2 \cdot k \cdot T \cdot V^0 \cdot t_R}{t^0 \cdot Vc \cdot w^2 \cdot \pi \cdot \eta} \tag{3}$$

where k is the Boltzmann constant and, T the temperature and, η the eluent viscosity.

This technology is often coupled with multi-angle light scattering (MALS) detector which makes possible the determination of absolute molar mass and size of the fractionated molecules from AFlFFF.

2.2. Multi-Angle Light Scattering

Theory and principle of MALS is described in literature (Wyatt, 1993; Zimm, 1948). In solution, macromolecules scatter light in all directions and the scattered intensity depends on many parameters in particular the scattering angle, the molar mass and the radius of gyration (Andersson, Wittgren, & Wahlund, 2001). One of the relations which exists between these parameters is described by Zimm equation:

$$\frac{K \cdot c}{R_\theta} = \frac{1}{M_w P(\theta)} + 2A_2 \cdot c \tag{4}$$

where K represents the optical constant, c is the concentration of macromolecules, M_w is their weight average molar mass, A_2 the second virial coefficient, and $P(\theta)$ is the scattering form factor. R_θ is the excess Rayleigh ratio which is function of the intensity measured at different angles according to

$$R_\theta = \frac{\left(I_{\theta(BP)} - I_{\theta(S)}\right) \cdot r_D^2}{I_0 V_0} \tag{5}$$

where I_θ is the scattering intensity of biopolymer solution (BP) and the solvent (S) at θ, I_0 is the intensity of the incident radiation, r_D is the distance between detector and scattering volume and V_0 is the scattering volume. $P(\theta)$ in Eq. 4 depends of the size and shape of the molecule and describes the angular dependence of the intensity of scattered light.

$$P(\theta) = \frac{1}{1 + \frac{16\pi^2}{3\lambda^2}\langle r_g \rangle^2 \sin^2\left(\frac{\theta}{2}\right)} \tag{6}$$

Coupled with fractionation technology (AFlFFF), the system is so diluted that c can be neglected. Eq. 4 can be written as $K \cdot c/R_\theta = 1/M_w P(\theta)$.

If extrapolated at zero angle ($P(\theta) = 1$), the molar mass and the radius of gyration could be obtained from Zimm plot of $K^*c/R(\theta)$ vs. $\sin^2(\theta/2)$ (Fig. 3).

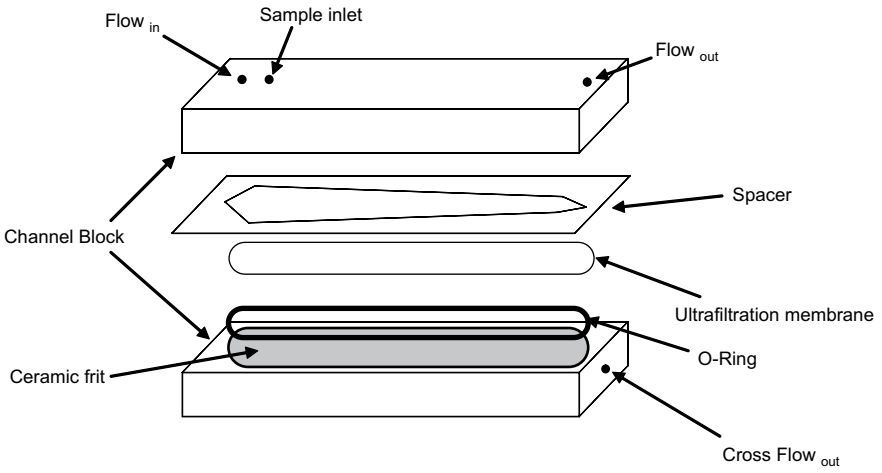

Fig. 1. Schematic representation of the AFlFFF channel.