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Evaluation of Molecular Weight Distribution of Sericin in Solutions Concentrated via Precipitation by Ethanol and Precipitation by Freezing/Thawing

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Sericin is a globular water-soluble protein that constitutes 25 to 30 % of silk proteins weight in silkworm`s cocoons. This protein envelopes silk fibers gluing them and ensuring the cohesion of the cocoon. During raw silk production, sericin is separated from silk fibers and it is discarded in wastewater, leading to a high biological oxygen demand to be degraded. The sericin molecular weight ranges from 10 to 400 kDa and the structure and molecular weight depend on the method which is extracted and purified. Lower molecular weight sericin peptides are mainly used in cosmetic products, whereas higher molecular ones enable its use for many other applications, like, medical biomaterials, functional bio membranes, and others. This study evaluates the molecular weight distribution of sericin in solutions in which this protein was concentrated by precipitation by ethanol and precipitation by freezing/thawing. The solutions, which were subjected to precipitations, were filtered and the two solutions obtained (the concentrated one and the permeated one) had their molecular weight distribution analyzed. The sericin molecular weight distribution in the solutions was analyzed by size exclusion chromatography (SEC). In this analysis, the mobile phase is ultra-pure water (Milli-Q) and a standard curve was made with pullulan polysaccharide standards. The results showed that the degumming process in autoclave (1 kgf/cm^2 – 40 min) was able to extract high molecular weight sericin and both precipitation methods could concentrate sericin in solution with no significant change in the molecular weight distribution, which ranges from lower than 20 kDa and up to 400 kDa values.

1. Introduction

Sericin is a protein present in the cocoon of the silkworm that deposited in layers on the fibroin fiber, maintains the cohesion of the cocoon by gluing silk threads together (Zhang, 2002). During the silk manufacturing, sericin is removed from the cocoons by a degumming process and it is usually discarded in wastewater. Annually worldwide, it is estimated that in those processes 50,000 tons of sericin are produced and not recovered (Zhang, 2002).
Sericin is a hydrophilic macromolecule that is made of 18 amino acids most of which have strongly polar side groups such as hydroxyl, carboxyl, and amino groups (Zhang, 2002).This composition enables easy cross-linking, copolymerization and blending with other polymers to form improved biodegradable materials (Dash et al., 2009). In addition, other characteristics including biocompatibility, antibiotic-antibacterial activity, antioxidant behavior, moisturizing capabilities and others (Aramwit et al., 2012), arouse interest in the development of this protein-based materials. These materials include healthy products, biomaterials and scaffolds for tissue engineering (Altman et al., 2003), films (Turbiani et al., 2011), functional membranes, hydrogels, functional fibers (Zhang, 2002) and materials for environmental

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purposes, like biosorption (Chen et al., 2012). Many researches look for alternative sources of biodegradable adsorbents to be used in biosorption processes, because it can provide an economical alternative for the removal of toxic heavy metals (Lima et al., 2013), dyes (Pelosi et al., 2013; Chen et al., 2012) and other pollutants from industrial wastewater.

The biological properties, composition and molecular size of sericin depend on the way it is obtained. Aramwit et al. (2010) demonstrated that silk extraction method in acid, alkali and urea solutions, affect physical (thermal behavior) and biological properties (tyrosinase inhibition); and Kurioka et al. (2004) also reported differences in trypsin inhibitor activity when the extractions were done in aqueous, acid and alkali solutions. The large range of molecular weights of sericin, 10 (Aramwit et al., 2012) to over 400 kDa (Gimenes et al., 2014), depends on the extraction methods, temperature, pH and processing time (Tomadon et al., 2010). Takasu et al. (2002) characterized the sericin components of fresh silkworm cocoons dissolving the protein in saturated aqueous lithium thicyanate containing 2-mercaptoethanol. It was found three major polypeptides whose molecular weight were 400, 250 and 150 kDa estimated by SDS-PAGE, which corresponds to the sericin present in the middle, anterior, and posterior part of the middle silk gland. Aramwit et al. (2010) evaluating extraction processes found that heat and acid extraction gives sericin a molecular weight of 35 – 150 kDa, whereas sericin extracted by alkaline solution has a molecular weight of 15 – 75 kDa.

The objective of this work is to evaluate the molecular weight distribution of sericin in concentrated solutions via precipitation by ethanol and precipitation by freezing/thawing. The sericin was extracted by using an autoclave at temperature of 120 ºC and it was subjected to precipitation processes and after the molar weight distribution was analyzed in size exclusion chromatography.

2. Materials and Methods

The *Bombyx mori* silkworm´s cocoons were kindly provided by Bratac Silk Mills Company, located in the State of Paraná – Brazil.

2.1 Preparation of cocoons and degumming process

The cocoons were manually cleaned and cut into small pieces (about 1 cm^2). Subsequently, they were carefully washed with tap water and rinsed with deionized water for three times. The cocoons were dried overnight at 50 °C, weighed and immersed in ultrapure water, in the ratio 3:100 w/V, in order to be used in the degumming process. The aqueous sericin solution (SS) was extracted using an autoclave at 120 °C (1 kgf/cm^2) for 40 minutes. The processing time started to be measured after the system had reached the desired temperature and pressure. The SS was vacuum filtered to remove the fibers from the solution, stored in a sealed container, and then it was maintained at room temperature for at least 12 hours to stabilize the hydrogel.

2.2 Precipitation Methods

In order to obtain a higher concentration of sericin solution, the SS extracted was subjected to two processes of precipitation: precipitation by ethanol and precipitation by freezing/thawing.

In the ethanol precipitation (EP), the SS was slowly poured over the same volume of ethanol and then the mixture was maintained in a freezer at 4 °C for 24 h to improve the precipitation and minimize the denaturation of the protein in ethanol (Wu et al., 2007). The system was not stirred during this period. The mixture was vacuum filtered and ethanol was removed from the two solutions, both the concentrated one and the permeated one, in a rotary evaporator operating at 50 °C with cooling water in the condenser at 10 °C. The samples were frozen and posteriorly analyzed on size exclusion chromatograph.

In the precipitation by freezing/thawing, the SS was frozen in a conventional freezer for 24 hours and then it was thawed at room temperature. The precipitated sericin was vacuum filtered and the concentrated and permeated solutions samples were frozen until the analyzes were done at size exclusion chromatograph.

2.3 Size Exclusion Chromatography Analyzes (SEC)

The Merck Hitachi LaChrom chromatograph was used in analyzes of molecular weight distribution of sericin. The analytical chromatographic system consisted of a quaternary pump L-7100, an automatic injector G-7250, a refractor index detector L-7490 and a module of heater column L-7300. The injection volume was 10 µL. The column, Ultrahydrogel Linear (300 mm x 7.8 mm id) Waters brand, was maintained at a temperature of 80 ºC. The mobile phase used was ultra-pure Milli-Q water at a flow rate of 1.0 $mL.min^{-1}$. The standard curve was built using pullulan polysaccharide standards of known and narrow molecular weight values, as follows: 10 kDa, 22.4 kDa, 47.2 kDa, 112 kDa, 212 kDa, 404 kDa, 788 kDa. These standards and the samples of sericin were injected at a concentration of 0.1% in water. The results obtained in this study were analyzed using the software Origin 8.0.

3. Results and discussion

To analyze the molecular weight distribution of precipitated sericin by ethanol and by freezing/thawing, initially, the calibration curve was made from standards pullulans and the curve fitting is shown at Figure 1. The known molecular weights and the retention times, obtained in chromatographic analyzes, of each pullulan were plotted.

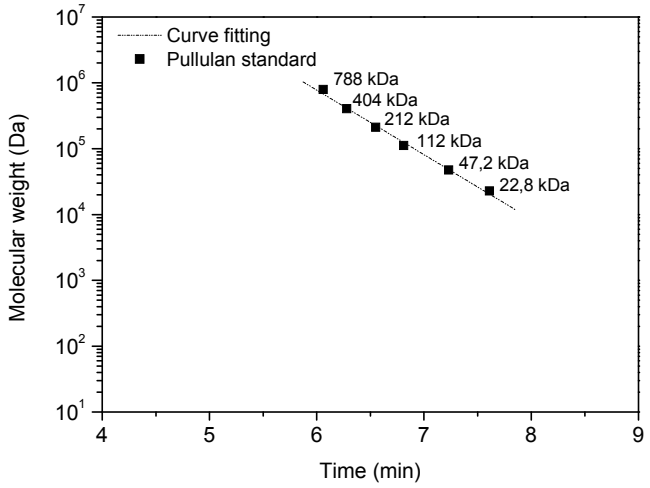

Figure 1: Molecular weight distribution of standard pullulan solutions (injection of 10 µL at a concentration of 0.1 % w/V of each analytical standard in Ultrahydrogel Linear (300 mm x 7.8 mm column id) Waters brand with ultrapure water as mobile phase at a flow rate of 1.00 mL/min and conditioned at 80 °C).

With the results obtained in the calibration step, it was possible to set the adjustment equation data: molecular weight (MW) *versus* time (t) (Eq. 1) with a correlation coefficient (R^2) of 0.982.

$$\log(MW) = -0.981 \times t + 11.773 \quad (1)$$

Figure 2 shows sericin molecular weight distribution in solution extracted by degumming process in autoclave. The pullulan standard curve is superimposed on the graphic.

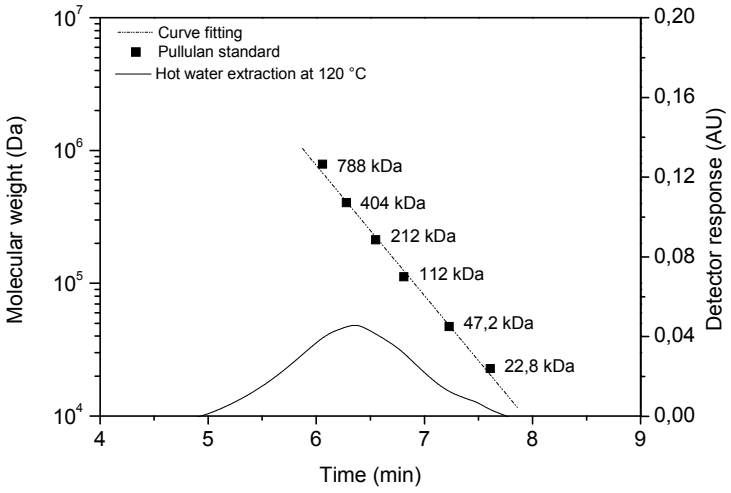

Figure 2: Molecular weight distribution of sericin in extracted solution in autoclave and the standard pullulan curve calibration.

The values observed in SEC analyzes, Figure 2, show a wide range of molecular weight values. Molecular weights were observed measuring from lower than 20 kDa to over 400 kDa. The results showed that the degumming process, in these analyzed conditions, was capable to solubilize high molecular weight sericin. The degumming process performed at major pressure and temperature, as extraction by autoclaving, promotes an increase in the extraction efficiency of sericin because the nearest sericin layers of fibroin fibers were solubilized. The sericin internal layers present higher molecular weight and major quantities of β-sheet structure in its secondary structure. According to Dash et al. (2009), Tomadon (2011) and Padamwar and Pawar (2004), secondary sericin structure is a largely random coil, which allows the solubility of these proteins in hot water, with some β-sheet structure, which makes it difficulty the solubility of the sericin, mainly in the nearest layers of fibroin fibers.
Figure 3 shows the molecular weight distribution of sericin solutions precipitated by ethanol. The two obtained solutions, the concentrated one and the permeated one, had their data plotted superimposed on the standard calibration curve of pullulans.

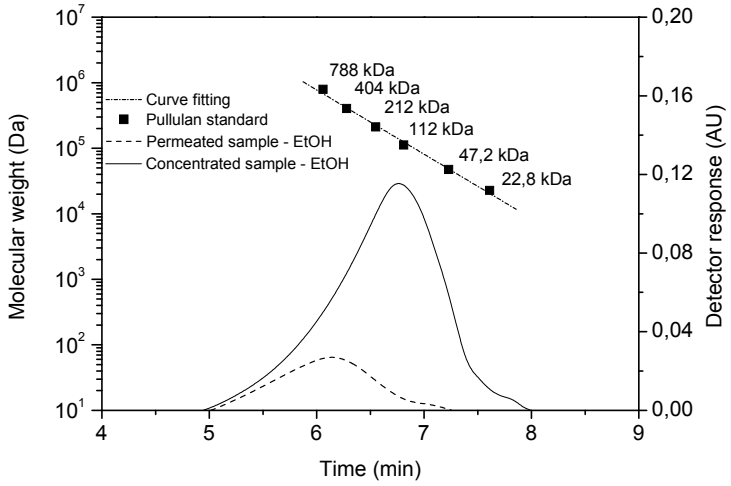

Figure 3: Molecular weight distribution of sericin in solutions precipitated by ethanol and the standard pullulan curve calibration.

In Figure 3, the chromatographic analyzes of the molecular weight of sericin in the permeated solutions and concentrated ones indicate a large range of distributions for both solutions. In the molecular weight distribution of concentrated solution, the values range from lower than 20 kDa and up to 400 kDa.
In the permeated solution, the molecular weight distribution was slightly lower. The values range from lower than 50 kDa (calculated by Equation 1, with a retention time of 7.21 min) and greater than 400 kDa, indicating a distribution of higher molecular weights values in the permeated solution when compared with the ones obtained with the concentrated solution.
According to Wu et al. (2007), sericin is a kind of natural water-soluble protein that is maintained in solution by surface hydrophilic groups with the solvent water, but adding ethanol that is less polar than water could reduce the polarity of the solvent and sericin tended to become less soluble, allowing the precipitation. Despite the loss of sericin that is not agglomerated (in permeated solution), it was possible to obtain a high rate of protein precipitation using ethanol precipitation procedure.
Figure 4 shows the molecular weight distribution curves obtained from the solution by precipitation using the process of freezing, followed by thawing. In the graphic, the curves of the concentrated solution, the permeated one and the standard curve pullulan are displayed.

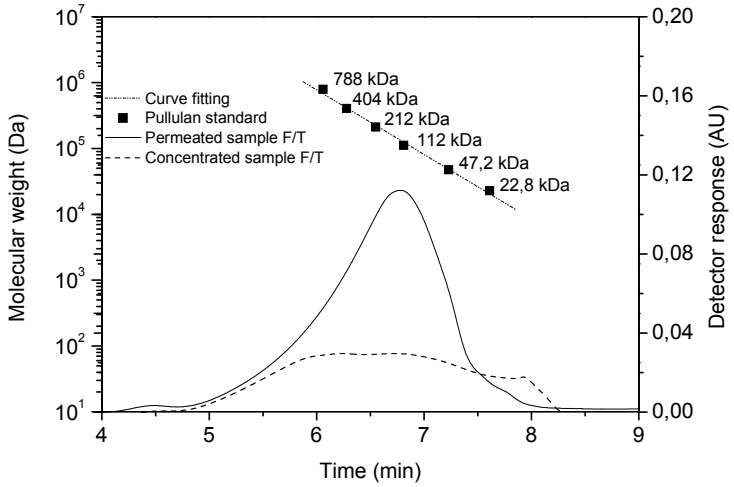

Figure 4: Molecular weight distribution of sericin solutions precipitated by freezing/thawing (F/T) and the standard pullulan curve calibration.

Figure 4 shows that the molecular weight distributions of sericin in the concentrated and permeated solutions were similar. In both curves, the distribution profile indicated molecular weight values lower than 20 kDa and up to 400 kDa. The globular proteins, as well as sericin, when frozen lose water to the surface of ice crystals, causing the destabilization of secondary and tertiary structures which promotes the formation of agglomerates or partial denaturation of the protein (Sgarbieri, 1996). The formation of agglomerates and partial denaturation makes it impossible to complete reabsorption of water, i. e., total solubility of the protein due to changes introduced in peptides (Tomadon, 2011; Sgarbieri, 1996), which allows the precipitation of sericin and its separation by vacuum filtration after being thawn.
The application of these precipitation methods on an industrial scale involves spending on lowering the temperature of the solution in both processes. In precipitation by freezing/thawing is necessary that the temperature be lower than 0 ºC, while in the precipitation by ethanol (at 4 ºC) a step of ethanol recovering (by distillation) is required so that the process becomes viable. Also, ethanol is an ecofriendly alcohol because it comes from renewable sources.

4. Conclusions

The analyzes of molecular weight distribution of sericin in the extracted solution by autoclaving (at 120 °C and 1 kgf/cm^2) showed that the degumming process was conducted under conditions capable to extract high molecular weight sericin, which is present in the layers nearest fibroin fibers. The precipitation processes by ethanol and freezing/thawing were able to promote the separation of proteins in solution without causing any significant change in the molecular weight distribution profiles of sericin in such solutions. The results were similar profile showing a distribution ranging from lower than 20 kDa and up to 400 kDa values.

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