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Study of thermal and chemical effects on cellulase enzymes: Viscosity measurements

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ABSTRACT

The behaviour of cellulase enzymes in phosphate saline buffer has been studied over a wide range of temperatures and enzyme concentrations by using viscosity measurements. To characterize the conformation change of cellulase versus temperature and chemical denaturants, such as guanidinium chloride (GdmCl) and urea, the information about the intrinsic viscosity and the hydrodynamic radius are necessary. The dependence of the intrinsic viscosity and the hydrodynamic radius in its random coil conformation on temperature and denaturant concentration were studied. Our results and discussions are limited to the dilute regime of concentration because of abnormalities in conformation observed in the very dilute regime due to the presence of capillary absorption effects.

1. Introduction

Protein physics stands on three fundamental experimental facts, namely, their three-dimensional structures [1,2], their native and unfolded states [3,4] and their aptitude to self-organize spontaneously during transition to their final solid ("native") 3D structures [5]. Therefore, several experimental and theoretical techniques have been used to investigate the process of protein folding and to determine fundamental factors allowing numerous biotechnological applications [6–14]. One of the simplest and most-effective techniques for studying the protein folding problem is the viscosity measurements [15]. The viscosity is a physical quantity that depends on the molecular weight, concentration, size, flexibility and intermolecular interactions [16]. The viscosity measurements are also interesting for studying the effects of temperature, denaturant concentration and pH on the protein denaturation [17]. In this study we aim to follow the conformational change of cellulase enzymes by using viscosity measurements. Cellulases are members of the large family of glycosyl hydrolases (GHs) [18]. They are generally secreted and act as endoglucanases (EGs), cellobiohydrolases or β-glucosidases [19]. Cellulases catalyse the hydrolysis of cellulose, the world's most abundant organic compound. They are also found in a variety of bacteria, fungi, plants and animals. Celluloytic enzymes play an important role in natural biodegradation processes of plant lignocellulosic materials. In industry these enzymes have found novel applications in the textile industry [20], brewing, paper pulp [21] and detergent industries [22]. Cellulases and related enzymes are necessary to increase the efficiency and economics of a wide range of biotechnological processes. However, the poor knowledge of their mechanism has lead to a real problem for their efficient industrial use for the conversion of plant material wastes into simple sugars. The ability of cellulases to degrade cellulose is central to the biological recycling of photosynthetically fixed carbon in the biosphere. Hence, due to the refractive nature of cellulase substrates (cellulose is an insoluble crystalline polysaccharide), cellulases have developed a modular organization characterized by a large catalytic module linked to a smaller cellulose binding module [23–25]. With few exceptions, the cellulose binding module is connected to the catalytic via highly glycosylated (in fungi) [26]. Therefore, the structural properties of the linker peptide of cellulases determine their action modes on the activity of cellulases by reducing the enzymatic activity on crystalline cellulose [27–29]. Small-angle X-ray scattering experiments applied to study cellulases conformational change have shown that the cellulase linkers are flexible and extended (for more details see Ref. [30]).

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This work focused the thermal and chemical denaturation effects on the conformational change of cellulases by using viscosity measurements. The conformational change of these enzymes was studied by determining enzyme intrinsic viscosity and hydrodynamic radius for temperatures varying between 27 and 55 °C. Our results and discussions are limited to the dilute regime of concentration because of abnormalities in conformation observed in the extremely dilute regime due to the presence of capillary absorption effects.

2. Experimental part

2.1. Sample preparation

The enzyme used in the course of this study was the cellulase from *Aspergillus niger* (EC. 3.2.1.4) with $M_r = 31{,}000$Da (Sigma-Aldrich, Germany). It was dissolved in 0.1 M phosphate buffer containing 0.134 M NaCl, in order to reduce the electrostatic interactions inside the solution, and 0.1 M of EDTA. The pH of the solution was adjusted to 7. The cellulases concentration was determined by the modified Bradford assay using bovine serum albumin (BSA) as standard [31–32]. The cellulases absorbance was measured at 450 nm and the concentration is obtained using the formula $absorbance = \varepsilon cl$ where c is the cellulases concentration, ε is the extinction coefficient and l is the path length (1 cm). The various concentrations used in this work were 6, 8, 10, 20, 30, 40, 50 and 60 mg/ml and were obtained by successive dilutions.

2.2. Viscosity measurements

An Ubbelohde capillary viscometer (Schott instruments) was used for the determination of viscosity. The capillary diameter of the viscometer was 0.4 mm. All the measurements were performed for different temperatures. The temperature control was assured by the incubation of the capillary inside a temperature-controlled water bath with an accuracy of 0.05 °C. To determine the viscosity of a sample, we first measured the flow time t_0 of the pure solvent for different temperatures. After this, we performed measurements of the flow time t of the sample in solution for the same set of temperatures and varying concentrations. Having t and t_0, the relative viscosity of the sample is determined by the following equation [33]:

$$\eta_r = \frac{t}{t_0} \tag{1}$$

The capillary used to measure the flow time of pure solvent was the same used for measuring the flow time of the sample solutions. This process is necessary because if the capillary used is different, we must introduce the capillary effects and the flow time becomes incorrect. In order to determine the specific viscosity of cellulase, we use the following equation:

$$\eta_{sp} = \eta_r - 1 \tag{2}$$

The time efflux measured for the capillary viscometer is related to the reduced viscosity of cellulases solution by the following formula:

$$\eta_{red} = \frac{t - t_0}{t_0 c} = \frac{\eta - \eta_0}{\eta_0 c} = \frac{\eta_{sp}}{c} \tag{3}$$

where, c the cellulases concentration, η_{sp} the specific viscosity, η_0 the solvent viscosity and η the viscosity of cellulases solution. If the reduced viscosity approaches zero, the intrinsic viscosity, representing the hydrodynamic volume occupied by a molecule, was given by Brandrup and Immergut [33,34]:

$$[\eta] = \lim_{c\to 0}\eta_{red} = \lim_{c\to 0}\frac{\eta_{sp}}{c} = \frac{\lim_{c\to 0}\eta_r}{c} \tag{4}$$

The intrinsic viscosity is obtained by the extrapolation of the reduced viscosity to zero cellulases concentration using the Huggins equation [33,34]:

$$\frac{\eta_{sp}}{c} = [\eta] + k_H[\eta]^2 c \tag{5}$$

where k_H is the Huggins coefficient obtained from the slope of the curve of the reduced viscosity.

3. Results and discussions

3.1. Temperature effects

To know the effects of temperature and enzyme concentration on viscosity, various concentration values (6, 8, 10, 20, 30, 40, 50 and 60 mg/ml) and a range of temperature varying between 27 and 55 °C have been tested. All used samples were prepared and measured in the same conditions.

Using Eqs. (1) and (2), we plot in Fig. 1 the variation of the specific viscosity η_{sp} for various temperatures versus cellulases concentration. From the curves we observe that the specific viscosity does not have a linear variation and those three regimes, characterized by three different slopes, appear. The first regime was observed for the range of concentrations between 6 and 10 mg/ml, the second between 20 and 50 mg/ml and the third is for concentrations above 50 mg/ml. We noticed that for concentrations <6 mg/ml the capillary effects have a great influence on the viscosity measurements [35,36]. For this reason, we limited our study to concentrations above 6 mg/ml. To identify these regimes, we take into account the Charpentier results [37] where he reports that the overlapping concentration c^*, which is the upper limit of the dilute regime. For this reason, we consider that the slopes (1), (2) and (3) characterize the extremely dilute regime, the dilute regime and the semi-dilute regime, respectively. On the other hand, the enhancement of the specific viscosity values is due to the suppression of the intermolecular electrostatic repulsion (presence of 0.134 M of NaCl) leading to the diminution of the coil dimension. For this

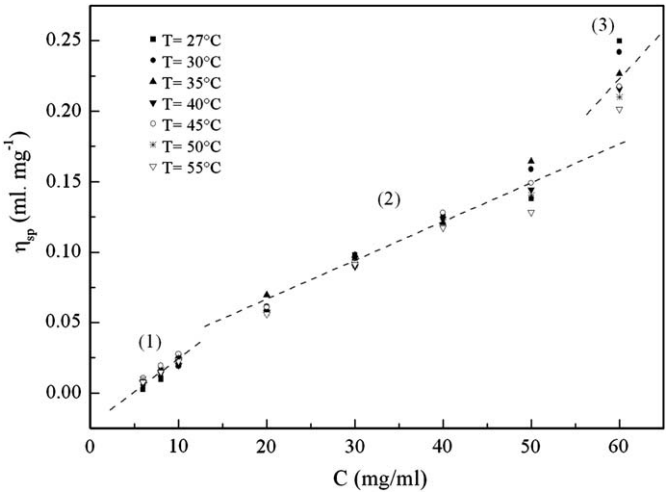

Fig. 1. Influence of cellulase enzymes concentration on the specific viscosity for various temperatures. The curve is characterized by three slopes representing different regimes: (1) extremely dilute; (2) dilute; and (3) semi-dilute regime.