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Investigation on curdlan dissociation by heating in water

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ABSTRACT

This study aimed to investigate molecular aggregation of curdlan on heating in water through atomic force microscopy (AFM), transmission electron microscopy (TEM), turbidity, and rheology tests. With improved sample preparation methods, AFM and TEM analysis revealed that curdlan morphologies changed with heating temperatures. Bundles of curdlan triple-stranded helixes had mainly hydration and swollen process at 40 °C, and bundled triple-stranded helixes were driven to separate from each other at 50 °C. These triple-stranded helixes would dissociate into partially opened triple-helical chains and single-helical chains at 60and 70 °C, and finally large amounts of dissociated single-helical chain were presented at 80 and 90 °C. Accordingly, structures of curdlan at temperatures ranging from 25 to 90 °C were proposed, and the turbidity and rheology results could be well explained with proposed structures. This research may provide new insights on revealing the behaviour of curdlan water dispersions on heating in water alone.

1. Introduction

Curdlan is an extracellular microbial polysaccharide fermented from *Alcaligenes faecalis* (Maeda, Saito, Masada, Misaki, & Harada, 1967). It is used in numerous food preparations as a food additive and has potential for biomedical and pharmaceutical applications due to its unique rheological properties and inherent bioactivity (Zhang & Edgar, 2014). Its degradation and derivatization products also showed high biological activity and could be used in biomedical fields, e.g. β-(1,3)-glucan oligosaccharides (Kumagai, Okuyama, & Kimura, 2016), phosphorylated and sulfonated curdlan (Kagimura, da Cunha, Barbosa, Dekker, & Malfatti, 2015).

Curdlan is a simple non-branching linear polysaccharide consisting of glucose residues linked by β-1, 3-glycosidic bonds. It has two conformations under different conditions. According to X-ray and ^{13}C NMR spectroscopy results, swollen curdlan (hydrous form) was mainly composted of single-stranded helixes, and annealed curdlan (both anhydrous and hydrous form) consisted of triple-stranded helixes (Deslandes, Marchessault, & Sarko, 1980; Saito, Yokoi, & Yoshioka, 1989; Takeda, Yasuoka, Kasai, & Harada, 1978). Besides, the long linear chain exists in the primary structure of curdlan, it can be changed to a rigid coil-like conformation in aqueous NaOH and dimethyl sulphoxide (DMSO) (Jin, Zhang, Yin, & Nishinari, 2006b). The molecular conformations of curdlan in aqueous NaOH and DMSO have already been studied. However, the unique behaviour of curdlan in water, which has interested many researchers since last century, has still some uncertainty.

Curdlan is insoluble in cold water, but can be dispersed in hot water, and forms two kinds of gel depending on the heating temperature. A low-heat reversible gel is formed when its dispersion is heated to 55–60 °C and then cooled below 40 °C, while highly thermally irreversible gel will be obtained when the dispersion is heated to 80 °C or above. Transformation of freezing bound water to non-freezing water was found when gelation mechanism is changed from low-set gel to high-set gel, indicating molecular structural changes (Hatakeyama, Iijima, & Hatakeyama, 2016). Based on X-ray diffraction results, the molecular structures changes of the two unique gels have been studies and discussed. For example, single-stranded helical structure was suggested on heating 4% aqueous suspension of curdlan at 70 °C for 10 min, and triple-stranded helical structure was observed when the same sample was heated above 120 °C (Okuyama et al., 1991). This triple-

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stranded helical structure was also reported on heating 2.0% aqueous suspension of curdlan at 95 °C for 10 min (Fulton & Atkins, 1980). The gelation mechanisms for this two different gelation process have been proposed. Using X-ray diffraction and infra-red spectroscopy, Fulton and Atkins (1980) proposed the gelling mechanism of curdlan as the association of micelles domains (formed by triple helices bounded with interstitial water), and not the untwining and retwining of single helices into triple stranded junction zones. Stipanovic and Giammatteo (1989) analyzed curdlan gels by ^{13}C NMR spectra, and suggested the conformation of curdlan in the low-set gel state at 60 °C was the seven-fold helical form, whereas a partial seven-fold form was transformed to six-fold triplex for a high-set gel formed at 95 °C. Saito, Yoshioka, Yokoi, and Yamada (1990) believed that the conformation of curdlan was mainly exhibited as a single helix with a low proportion of triple helixes in the low-set gel and a high proportion of triple helixes in the high-set gel, based on observation of high-resolution solid-state ^{13}C NMR spectra of curdlan in hydrate, anhydrous, annealed, and gel forms. Okuyama et al. (1991) studied the polymorphs of curdlan using X-ray diffraction and thermal analyses, proposing that single helices existed in the low-set gel and triple helices in the high-set gel. Ikeda and Shishido (2005) investigated molecular structures of the unheated and heat-treated curdlan dissolved into NaOH aqueous solutions using AFM. They suggested that the heat-induced gelation of curdlan appeared to be partial dissociation of single chains from entangled microfibrils, and the later cross-linking among these partially dissociated chains. Till now, the gelation mechanism of curdlan and the fine network structures along with conformation are still not well understood (Cai & Zhang, 2016).

Despite the detailed studies of molecular conformations, gel structures, gelation mechanisms, the behaviour of curdlan water dispersions on heating in water, and proposed helical conformation changes of curdlan water dispersions on heating in water, there still lack direct evidence for the exact molecular conformation of curdlan in water. Therefore, the aim of this study was to investigate molecular aggregation of curdlan on heating in water by microscopic observation with improved methods for sample preparations. The changes on turbidityand rheological properties of curdlan water dispersion were also investigated and discussed. This research would contribute to deeper understandings on the behaviour of curdlan water dispersions on heating in water.

2. Materials and methods

2.1. Materials

Curdlan sample (purity $\geq$ 95%) was purchased from Shandong Zhongke Biological Technology Co., Ltd. (Weifang, China), and used without further purification. The viscosity average molecular weight of curdlan was 1.1×10^6 g/mol in aqueous NaOH.

2.2. Microscopic observation of curdlan on heating in water

2.2.1. Improved methods for heated curdlan sample preparation for microscopy

In previous reports, the conformation of curdlan was observed in aqueous NaOH. For example, Harada, Koreeda, Sato, and Kasai (1979) heated curdlan water dispersion, and deposited it on a grid before air-dried at room temperature, and observed by TEM. Ikeda and Shishido (2005) heated curdlan in aqueous NaOH, and then deposited diluted sample onto a mica surface before air-dried at room temperature, and observed by AFM. These methods only allow the conformation to be observed at room temperature, and are not persuasive enough to exactly demonstrate the morphology of curdlan under heated condition, as curdlan molecules underwent aggregation changes on heating which could be changed again on cooling (Hirashima, Takaya, & Nishinari, 1997). Moreover, most researchers adopted relatively high samples concentration (e.g. 0.05%, w/v and 1.0%, w/w in above references) to heating treatment before dilution, which may undergo the reversible and irreversible gelation process, and their real morphology could be concealed.

Therefore, we have made two improvements in the method of sample preparation. First, the diluted curdlan water dispersions with extremely low concentration (2.5 μg/mL) were prepared before heating treatment, rather than heating concentrated curdlan water dispersions, and then observed by both AFM and TEM. Second, the diluted curdlan was prepared at a series of temperatures (40–90 °C) and also dried at these temperatures to avoid any molecular structural changes of curdlan during drying. The detailed procedures are described in the following section.

2.2.2. Preparation of samples for microscopy

Curdlan water dispersion (0.5 mg/mL) was firstly prepared by dispersing curdlan powder (0.01 g, dry basis) into 20 mL MilliQ water in a vial bottle, with stirring using a digital multi-point magnetic stirrer (MS-MP8, DAIHAN Scientific, Seoul, Korea) at 450 rpm at 25 °C for 12 h. The vial bottle has a threaded neck and is sealed with plastic threaded cap. Then the highly diluted curdlan water dispersion (2.5 μg/mL) was prepared by pipetting 50 μL above curdlan dispersion into a new vial bottle containing 10 mL MilliQ water, with still stirring at 450 rpm at 25 °C for a few minutes. Samples were then put into a constant temperature magnetic stirring apparatus (DF-101S, YUHUA Instruments, Gongyi, China) at heating temperature 40, 50, 60, 70, 80, and 90 °C at 450 rpm for 2 h, and separately transferred into a constant temperature and humidity glove box (DHS-100, Jintan Jianwei Environment Instrument, Jiantan, China) at the same temperatures. Then curdlan water dispersions (8 μL) were separately pipetted onto a TEM copper grid and a mica substrate. The micro pipette, micro pipette tips, TEM copper grid covered with porous carbon films, and the mica used here were all preheated to 40, 50, 60, 70, 80, and 90 °C in the glove box. After complete water evaporation, the copper grid and mica were taken out and transferred into a glass drying containers (with silica gel) for subsequent AFM and TEM tests.

2.2.3. Microscopic observation

Samples prepared in section 2.2.2 were negatively stained with 2.0% phosphotungstic acid before TEM test. They were observed using a transmission electron microscopy (JEM-2100, JEOL Ltd., Japan) with a Gatan 830 CCD camera at an accelerating voltage of 200 kV. The Gatan DigitalMicrograph (3.7 version) software was used to analyze the images. Samples were tested at least in triplicate and four images were obtained with each replication.

Atomic force microscopy (AFM) was performed using Nanoscope IIIa Multimode (Veeco Co., Santa Barbara, CA) equipped with an E-scanner. Tapping mode cantilevers with nominal spring constant of 5–100 N/m and nominal resonance frequencies of 10–320 kHz were employed. Processing of AFM images was performed with NanoScope software (NanoScope Analysis v140r1sr2). Samples were tested at least in triplicate and four images were obtained with each replication. The height of objects present in AFM images were analyzed by NanoScope software.

2.3. Turbidity measurement

Curdlan water dispersion (5.0 mg/mL) was prepared the same as described in section 2.2.2. Then the samples were put into the