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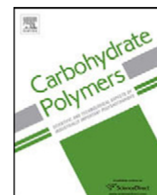

Effect of carboxymethyl groups on degradation of modified pullulan by pullulanase from *Klebsiella pneumoniae*

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ABSTRACT

Pullulanase is an enzyme that hydrolyses the α-1,6 linkages of pullulan (Pull) to produce maltotriose units. We studied the capacity of pullulanase to cleave its modified substrate: carboxymethylpullulan (CMPull), synthesized with two different degrees of substitution (DS = 0.16 and 0.8). Size exclusion chromatography with on line multi angle light scattering and differential refractive index detection (SEC/MALS/DRI) was used to estimate both number and weight average molar masses, respectively, Mn and Mw, of pullulan and CMPulls together with the percentage of maltotriose formed during hydrolysis. Determination of reduced sugars gave also a Mn that is compared to data obtained by SEC. It revealed that CMPull is partially degraded by pullulanase and the rate of hydrolysis decreased with increased DS. At the end of the hydrolysis, Mn is decreased by a factor of 23 and 1.7 for CMPull with a DS of 0.16 and 0.8 respectively. The percentage of produced maltotriose decreased also when increasing DS (24% and 7% for CMPull DS 0.16 and 0.8 respectively). The kinetic properties of pullulanase were also investigated with Pull and CMPulls by isothermal titration calorimetry (ITC) using simple injection method. Based on Michaelis–Menten kinetics, V_{max} (maximal velocity) decreased and K_M (Michaelis constant) increased when DS of modified pullulan CMPull increased.

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1. Introduction

Pullulan (Pull) is a neutral and biodegradable polysaccharide, composed of maltotriose units linked by α-1,6 bonds (Singh, Singh, & Saini, 2009; Teramoto & Shibata, 2006) and selectively hydrolyzed by pullulanase (EC 3.2.1.41) which can determine the chemical structure of polysaccharide and improve the production yield of many sugars (Kuroiwa, Shoda, Ichikawa, Sato & Mukataka, 2005; Mondal, Sharma, & Nath Gupta, 2003). Pullulanase is an enzyme whose hydrolyze the (1,6)α-D-glucosidic linkages in pullulan and amylopectin. It improves the saccharification of starch to produce glucose, maltose and malto-oligosaccharides using glucoamylase, β-amylase or α-amylase. The using of pullulanase is essential for efficient processing of starch in food industries (Kuroiwa et al., 2005). As well, the efficiency of conversion of starch and glycogen is increased by adding pullulanase in the medium containing amyloglucosidase. The glucose produced by hydrolysis can be used for the production of high fructose syrup or as a feedstock for microbial fermentations (Ajoy & Kenneth, 1990). In other hand, Pullulanase was used for the determination of glycogen structure and for determining the mechanism of actions of enzymes that synthesize glycogen (Walker, 1968). It was found that glycogen and amylopectin debranching enzymes, such as pullulanase and isoamylase can be used as effective additives in dishwashing and laundry detergents (Ara et al., 1995).

So, the interest in pullulanase arose mainly because of its application in structural studies of glycogen and amylopectin and because of its use as an industrial debranching agent.

One can distinguish in general two types of enzymes: Pullulanase of type I which hydrolyzes the α-1,6 glucosidic linkages in pullulan to obtain maltotriose as the end product and type II which hydrolyzes the α-1,6 glucosidic linkages of pullulan as well as α-1,4 glucosidic of other polysaccharides (Ara et al., 1995; Kimura & Horikoshi, 1990; Kunamneni & Singh, 2006; Repellin, Baga, & Chibbar, 2008). It is used in combination with other enzymes in industrial process for the production of sugar syrups (Singh, Saini, & Kennedy, 2010a, 2010b).

To search for new properties of pullulan, many studies have been reported on the chemical modification of pullulan such as: chloroalkylation (Mocanu, Vizitiu, Mihai, & Carpov, 1999), nitroalkylation (Heeres et al., 2000), alkyl etherification (Henni-Silhadi et al., 2007; Shibata, Nozawa, Teramoto, & Yosomiya, 2002). Carboxymethylation of pullulan, with the aim to obtain polyelectrolytes, may occur on the three hydroxyl functions of the two different sugars of maltotriose unit but the substitution of C-2 is predominant and decreases according to the order C-2 > C-3 > C-6 > C-4, an order of relative reactivity of hydroxyl groups as follows: $(OH)_2 > (OH)_4 > (OH)_6 > (OH)_3$ (Glinel, Sauvage, Oulyadi, &

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Huguet, 2000). Polyelectrolytes have many applications due to their unique physicochemical characteristics. Particularly, the carboxymethylpullulan (CMPull) has been described as well as their amphiphilic derivatives of various degree of grafted hydrophobic chains (Simon, Picton, Le Cerf & Muller, 2005) and it has interest in many applications in biotechnology and medicine (Mocanu, Mihai, Dulong, Picton, & Le Cerf, 2011). It has been also used as precursor to form polyelectrolyte pH-sensitive hydrogel by cross-linking reaction (Dulong, Mocanu, & Le Cerf, 2007).

Recently, it was shown that this polyelectrolyte is retained in the blood circulation and is expected to accumulate in a tumor because of passive targeting. It is expected to be a promising carrier for targeting immune tissues with an immunosuppressant to enable treatment of autoimmune diseases (Masuda et al., 2001). It was also shown that the system CMPull – ligand would be an excellent and novel carrier for targeting a drug specifically to inflammatory sites (Horie et al., 1999). CMPull can also be used with other polysaccharides to form magnetic nanowires structures using the layer by layer method. These nanostructures can be used for biological applications (Magnin et al., 2008).

However, the activity study of pullulanase on CMPull or on other modified pullulans was not reported. In this paper, we reported pullulanase activity data on CMPull with a DS of 0.16 and 0.8. We have determined the oligosaccharide fractions and the molar masses distribution by size exclusion chromatography (SEC) with on line multi angle light scattering (MALS) and differential refractive index detection (DRI) from enzymatic digests of Pull and CMPulls. The determination of kinetic parameters is obtained by isothermal titration calorimetry (ITC). The objective of this study is to gain an understanding of the relationship between Pull modification in term of substitution and the ability of pullulanase to degrade this modified polysaccharide.

2. Experimental

2.1. Materials

Pullulan was purchased from Hayashibara Biochemical Laboratory (Japan). Enzyme pullulanase from *Klebsiella pneumoniae* was produced by Sigma–Aldrich. Sodium chloroacetate was purchased from Sigma–Aldrich. Potassium sodium tartrate tetrahydrate, 99% for analysis, and isopropanol, extra pure were produced by Acros Organics. Lithium nitrate was obtained by VWR, 3,5-dinitrosalicylic acid was purchased from Fluka Analytical. All compounds were used without further purification.

2.2. Synthesis of carboxymethylpullulan

CMPull was synthesized in isopropanol/water medium (66/33, V/V) at 70 °C by addition of sodium chloroacetate which reacts with hydroxyl groups of pullulan in the presence of sodium hydroxide. After 4 h, the aqueous phase was neutralized, dialyzed and lyophilized. DS represents the carboxyl group number by anhydroglucoside unit (AGU) (Dulong, Le Cerf, Picton, & Muller, 2006). To study the influence of carboxylic group on the kinetics of hydrolysis by pullulanase, two CMPull's were synthesized with a DS of 0.16 and 0.8 respectively.

2.3. Determination of substitution degree

The DS of synthesized CMPull was determined by conductimetric titration by means of Eyler's method (Eyler, Klug, & Diephius, 1947).

A solution of CMPull was prepared by dissolving 0.225 g of CMPull in 50 mL of water. The polysaccharide concentration is determined by dried extract using 7 mL of this solution (the humidity rate is around 14%). The neutralization of all carboxylic acid functions is performed by addition of 1 mL of NaOH 1 mol L^{-1}. The titration was performed by conductimetric measurements using a solution of HCl at 0.1 mol L^{-1} in a reactor at 25 °C.

2.4. Enzymatic hydrolysis

The reaction mixture was prepared by mixing the enzyme solution (1 g L^{-1}) with substrate (Pull or CMPull) at 7.5 g L^{-1} in a solution of $LiNO_3$ 0.1 mol L^{-1} at 60 °C and pH 5.5. At fixed time intervals, different aliquots were taken. We performed 3 independent experiments for each hydrolysis.

A study with $LiNO_3$ 0.5 mol L^{-1} was performed with the aim to check if any specific interactions (mainly electrostatic ones) occur between CMPull and pullulanase.

2.4.1. Reducing sugars assay

The hydrolytic activity was estimated by determining the concentration of reducing sugars by 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959) which consists of preparing a solution by dissolving 30 g of potassium sodium tartrate tetrahydrate and 2.5 g of NaOH pellets with 1 g of 2-hydroxy-3,5-dinitrosalisilic acid in 50 mL water. The two solutions were combined and the resulting solution was diluted with 20 mL water. The amount of reducing ends in a hydrolyzate was determined by adding 2 mL of DNS reagent to 0.5 mL of hydrolyzate. The mixture was heated to 100 °C in a water bath for 5 min. After cooling, the absorbance at 540 nm was measured using a Perkin-Elmer Lambda 7UV/vis spectrophotometer (USA). Calibration was achieved using maltotriose. The uncertainty is close to 3%.

We have verified two points. A totally degradation of a pullulan known quantity gives the good value of reducing sugars using maltotriose calibration. The determination of reducing sugars of a CMPull DS 0.8 solution at high concentration shows that the calibration is correct for CMPull samples.

2.4.2. Determination of molar masses

Size exclusion Chromatography (SEC) with on line Multi-Angle Light Scattering (MALS) and Differential Refractive Index (DRI) detectors is a useful technique for determination of molar masses distribution analysis of polymers, and also for direct determination of the average molar masses. The most widely used SEC detector is the RI detector. Our versatile technique for molar masses distribution and average molar mass analysis of samples without calibration is SEC/DRI combined with MALS detection.

$LiNO_3$ 0.1 mol L^{-1} was used as carrier, filtered through 0.1 μm filter unit (Millipore), degassed (DGU-20A3 Shimadzu, Japan), eluted at 0.5 ml min^{-1} flow rate (LC10Ai Shimadzu, Japan). Before injection, each sample with pullulan and pullulanase was boiled at definite times to stop the enzymatic reaction. The hydrolyzed pullulan was then injected onto analytical line equipped with automatic injector (SIL-20A Shimadzu, Japan) at 100 μL. The SEC line consisted of an OHPAK SB-G guard column as protection and two OHPAK SB 802.5 and 804 HQ columns (Shodex Showa Denko K.K., Japan) in series. The MALS photometer, a DAWN-EOS from Wyatt Technology Corp. (Santa Barbara, CA) is filled with a K5 cell and 18 diode measurements (angle spread around the cell). The collected data were analyzed using the ASTRA V 5.3.4.18 software package using Zimm order 1. The concentration of each eluted fraction has been determined with the DRI (RID 10A Shimadzu, Japan) according to the known value of differential refractive index dn/dC (dn/dC = 0.14 mL g^{-1} Simon, Dugast, Le Cerf, Picton & Muller, 2003). The number and weight average molar masses of Pull and CMPull were determined by SEC/MALS/DRI.

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