Carbohydrate Polymers 93 (2013) 109–115

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

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

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ARTICLE INFO

Article history:
Received 17 October 2011
Received in revised form 9 July 2012
Accepted 11 July 2012
Available online 20 July 2012

Keywords:
Polysaccharide
Pullulanase
SEC/MALS/DRI
ITC
Hydrolysis
Enzyme kinetics

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.

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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http://dx.doi.org/10.1016/j.carbpol.2012.07.039

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.

2.4.3. Isothermal titration calorimetry

There are two approaches for studying enzyme kinetics by ITC: the single injection (continuous assay enzyme kinetics) method and the multiple injection (pseudo-first-order enzyme kinetics) method (Todd & Gomez, 2001; Olsen, 2006). It was shown that there was no significant product inhibition when using the single injection assay. In this work, the enzyme kinetics of pullulanase was investigated using simple injection method.

Our experiments were conducted using an ITC instrument (Nano ITC 2G, TA Instrument, UK). Pullulanase, Pull and each CMPull substrate were prepared in 0.1 M $LiNO_3$, pH 5.5. We performed three independent experiments for each sample with a very good repeatability. Enzyme kinetics experiment performed in the volume ITC with a fixed gold cell consisted of titrating 8.3 10^{-6} mol L^{-1} pullulanase (in syringe) into a solution of polysaccharide at 0.5 g L^{-1} (in the reaction cell). ITC experiments consisted of one simple injection, with stirring speed of 250 rpm at 60 °C. The reference cell was filled with $LiNO_3$ 0.1 mol L^{-1} solution. Blank test (i.e. $LiNO_3$ 0.1 mol L^{-1} pH 5.5 in the syringe) has been subtracted from experimental measurements. Data were collected with ITC Run data acquisition software and analyzed with Nano Analyze Software®. The thermal power is monitored continuously as the substrate is converted to product, and continues until the substrate is depleted and the thermal power returns to the initial baseline. A continuous curve is obtained when the rate is plotted as a function of substrate concentration normalized for the concentration of enzyme. Calculation of the reaction rate requires knowledge of the enthalpy change of the reaction (ΔH_r) and the volume (V) of the reaction chamber (Todd & Gomez, 2001). The enthalpy change is calculated by measurements of the total-heat and the total number of moles which are converted from substrate to product, Eq. (1):

$$\Delta H_r = \frac{1}{n_{sub}} \int_{t=0}^{t=\infty} \frac{dQ}{dt} dt \quad (1)$$

where n_{sub} is the number of moles of substrate which are converted to products and dQ/dt is the heat-flow. Thus, the reaction rate (ϑ) can be calculated by using Eq. (2):

$$\vartheta = \frac{1}{V\Delta H_r} \frac{dQ}{dt} \quad (2)$$

The substrate concentration ($[sub]_t$) as a function of time can be calculated using the integrated heat as a function of time, Eq. (3):

$$[sub]_t = \frac{\int_{t=0}^{t} (dQ/dt)dt}{\Delta H_r V} \quad (3)$$

3. Results and discussions

3.1. Monitoring degradation of Pull and CMPulls by SEC/MALS/DRI

The three polysaccharides (Pull and the two CMPulls) and the product of their degradation by pullulanase were analyzed using SEC/MALS/DRI method. As an example, chromatograms with both DRI and LS at 90° responses together with the molar masses distributions for Pull and a treated Pull at the very beginning of the degradation (1 min) are shown in Fig. 1. The exploitation of such data (Mn, Mw (number and weight average molar mass) and polydispersity index (PI)) is presented in Table 1 together with similar results obtained on untreated CMPull which are consistent with the literature (Simon et al., 2003).

Analysis of degraded polysaccharides is more delicate. We observed many populations resulting of the degradation. For the larger species (i.e. lower elution volumes), the LS response is sufficient to permit the calculation of molar masses. Some low size species that are probably oligosaccharides (here from 15 mL) do not have enough LS response. So it appears impossible to determine molar masses with good accuracy. This can be seen with the very bad results of molar masses in this area in Fig. 1. As a consequence the average Mn cannot be calculating using this lower values of molar masses and therefore should appear overestimated in comparison with Mn obtained from the reducing sugars analysis. Nevertheless, chromatograms of treated samples evidence three distinct DRI peaks at 17.2, 17.8 and 18.5 mL. An injection of maltotriose, the ultimate product of the enzymatic treatment, gives one peak at 18.5 mL (data not shown). Consequently, it becomes possible to measure the quantity of maltotriose (DP 3) in each sample thanks to the DRI response. As we use a pullulanase of type I, we can expect that the two other peaks (17.8 and 17.2) are fractions of DP 6 and 9. For lower elution volumes, we observe a DRI response which corresponds to higher DP than 9.

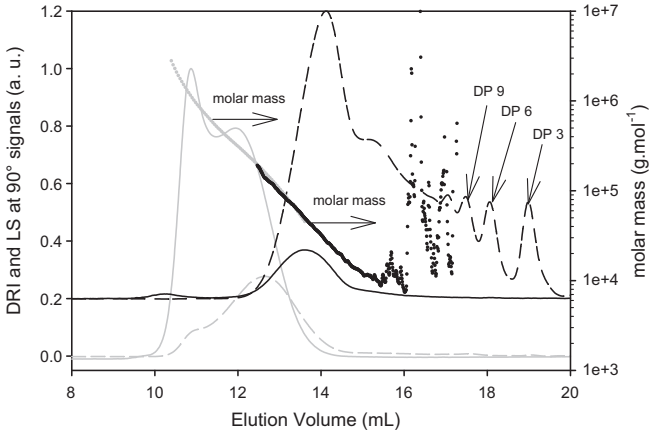

Fig. 1. Elution profiles obtained by SEC/MALS/DRI from refractive index (dotted lines) and LS (full lines) of non-degraded Pull (gray) and Pull treated 1 min by pullulanase (black) together with molar masses distribution in $LiNO_3$ 0.1 mol L^{-1} at pH 5.5.

3.2. Action of pullulanase on Pull and the two CMPulls: enzymatic hydrolysis

Pullulanase from *K. pneumoniae* hydrolyses the α-1,6 links of pullulan to produce maltotriose. It was examined for its ability to hydrolyze two various CMPull with DS 0.16 and 0.8 respectively at 60 °C and pH 5.5 in $LiNO_3$ 0.1 mol L^{-1}. The products of hydrolysis were followed and analyzed by chromatography (SEC/MALS/DRI) to determine the number and weight molar masses (Mn and Mw) of degraded polysaccharides as a function of time (Fig. 2). Pullulan appears quickly degraded and after 4 h we observed only one peak of maltotriose. Consequently we have attributed the theoretical value of 530 g mol^{-1} for both Mn and Mw (see stars in Fig. 2).

Kinetics of CMPull hydrolysis are difficult to discuss due to the small decrease of the molar masses during the pullulanase treatment. Enzymatic degradation conducted with CMPull 0.16 lead to Mn and Mw which are reduced with a factor 23 and 12 respectively. CMPull 0.16 is less degraded than the unmodified Pull indicating that the presence of carboxymethyl groups prevent the enzyme action. Reinforcing this observation, the results obtained for CMPull

Table 1
Molar masses of precursor Pull and two CMPulls 0.16 and 0.8 before hydrolysis by pullulanase, determined by SEC/MALS/DRI in $LiNO_3$ 0.1 mol L^{-1}.

	Mn (g mol^{-1})	Mw (g mol^{-1})	PI = Mw/Mn
Pull	120,000 (±1%)	240,000 (±2%)	2.0 (±2%)
CMPull 0.16	130,000 (±2%)	230,000 (±3%)	1.8 (±2%)
CMPull 0.8	140,000 (±1%)	230,000 (±1%)	1.7 (±1%)

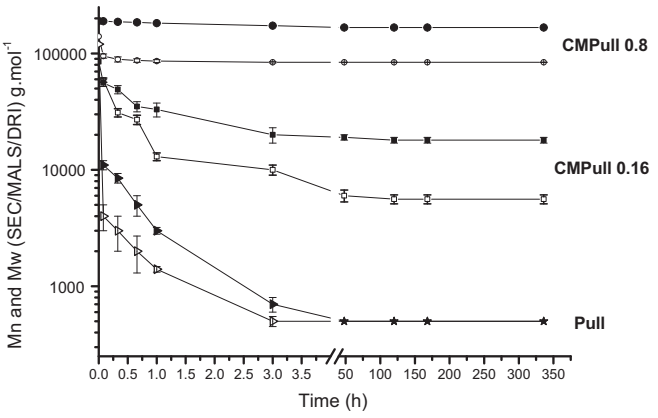

Fig. 2. Number (open symbols) and weight (filled symbols) molar masses determined by SEC/MALS/DRI as a function of time (h) for Pull (▲), CMPull 0.16 (■) and CMPull 0.8 (●) after hydrolysis by pullulanase and (⋆) theoretical molar mass of maltotriose (only one peak of maltotriose observed).

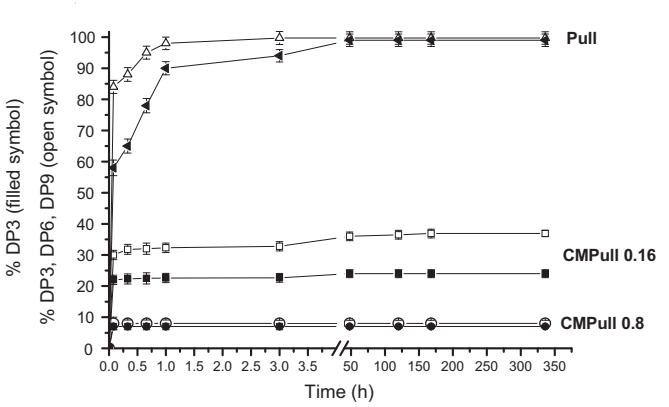

Fig. 4. percentage of formed maltotriose units (filled symbols) and oligosaccharides with DP 3, 6 and 9 (open symbols) versus time after hydrolysis of Pull (triangle) and CMPull with DS 0.16 (square), 0.8 (circle) by pullulanase *from Klebsiella pneumoniae*.

0.8 indicate a very slow and low hydrolysis since both Mn and Mw decrease only with a factor lower than 2 after the enzymatic treatment. It appears obvious that the presence of carboxymethyl groups on modified Pull makes it resistant to enzymatic hydrolysis.

On the other hand, we have determined the reducing sugar amount by the DNS assay and have calculated the corresponding average number molar mass Mn (Fig. 3) for Pull and the two CMPulls during the enzymatic treatment. The obtained results are logically consistent with the results obtained with SEC/MALS/DRI analysis evidencing the same tendency. The presence of carboxymethyl groups diminishes the enzymatic hydrolysis. As expected and explained above, Mn obtained from SEC/MALS are slightly overestimated as compared to the Mn obtained using the reducing sugar method. This can be related to the smaller oligosaccharides which cannot be determined by SEC/MALS/DRI measurements.

As evocated in Section 3.1, the chromatograms of degraded samples show the presence for maltotriose units. We have calculated the % of maltotriose units but also of oligosaccharides with DP 6 and DP 9 formed in each case. We have reported in Fig. 4, the amount of DP 3 and (DP 3, 6, 9) for the studied samples as a function of time. For the unmodified Pull, the final residues are essentially the maltotriose units (only one peak characteristic of DP 3) and logically some residues of DP 6 and 9 are obtained. For CMPull, the result suggests that an increase of the DS (i.e. the number of carboxymethyl group on Pull) decreased the % of maltotriose units generated by hydrolysis (24% for CMPull 0.16 and 7% for CMPull 0.8). This result is consistent with the limitation of enzymatic activity when the amount of carboxymethyl groups increases. The unmodified pullulan segments in the CMPull are logically longer and more accessible when DS decreases. Consequently, the hydrolysis of the most grafted CMPull (i.e. CMPull 0.8) lead to hardly DP6 and DP9 while it becomes possible to observe the three oligosaccharides with DP 3, 6 and 9 for CMPull 0.16.

The inhibition of the degradation can be explained by the presence of carboxymethyl groups but also by the loss of activity of the enzyme during long periods of times. For highlighting this last point, we have added after 24 h of hydrolysis another amount of enzyme in the medium. After 24 h, we have taken an aliquot that was analyzed by SEC/MALS/DRI. We obtained the same molar masses before and after the new addition of pullulanase. We can conclude now that the hydrolysis of CMPull is really dependent on the degree of substitution of carboxymethyl group.

Other studies have investigated the relationship between the resistance toward enzymatic degradation and the DS for starch (Steeneken et al., 2008), carboxymethylcellulose (Cohen et al., 2004), hydroxyethylcellulose (Martinez-Richa, 1998) and other cellulose derivatives (Glasser, McCartney, & Samaranayake, 1994; Mischnick, 2001; Murphy, Borch, McFarland, Bohlin, & Westh, 2010; Richardson & Gorton, 2003). As an example, the resistance of hydroxyethylcellulose to degradation occurs only for DS higher than 1.34. Nevertheless, it often appears that the resistance of substituted substrate toward enzymatic degradation depends on the DS but also on the distribution of substituted groups on the polymer chain. It may also depend on the enzymatic mechanism.

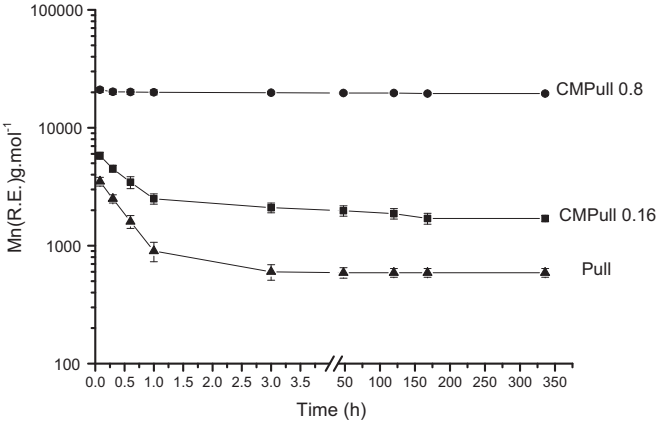

Fig. 3. Number molar mass determined with reducing extremities by DNS assay as a function of time (hours) for Pull (▲), CMPull 0.16 (■) and CMPull 0.8 (●) after hydrolysis by pullulanase.

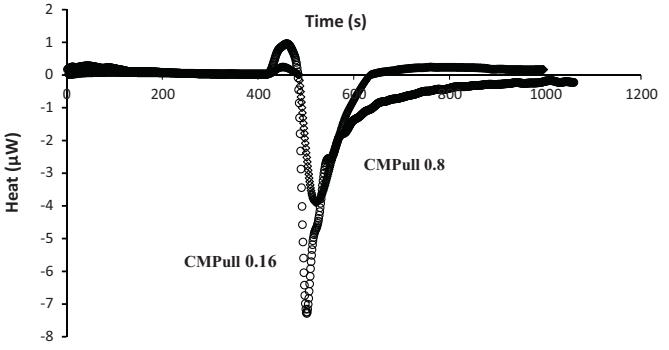

Fig. 5. Single injection assay: after 400 s, 250 µL of pullulanase at 8.3×10^{-6} mol L^{-1} was injected to the calorimetric cell raw data of a calorimetric experiment containing Pull (A), CMPull 0.16 and 0.8 (B) at 0.5 g L^{-1} in $LiNO_3$ 0.1 mol L^{-1}, pH 5.5 at 60 °C.

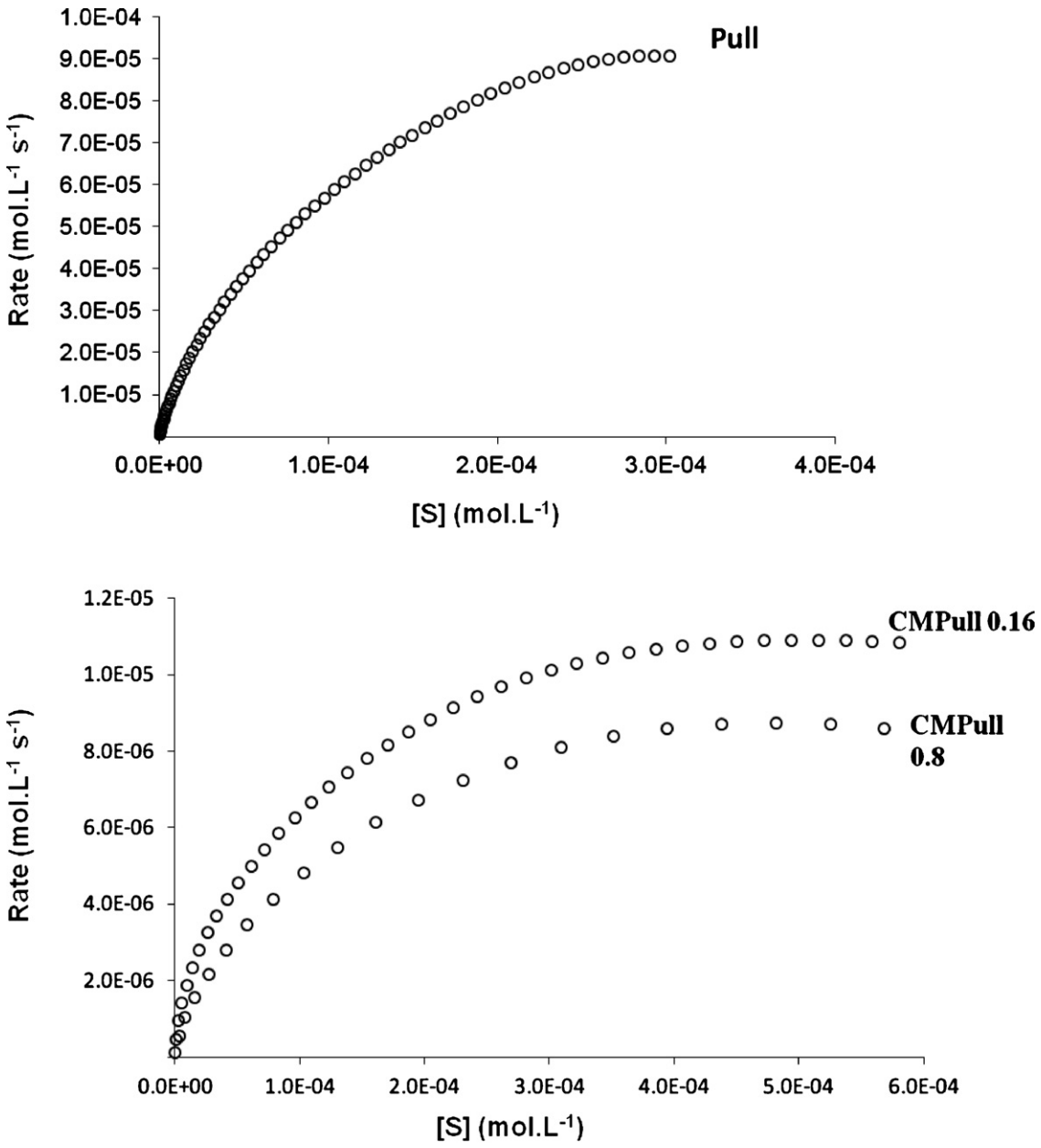

Fig. 6. Catalytic rate versus the maltotriose concentration for Pull (A), CMPull 0.16 and 0.8 (B).

3.3. Complexation

In literature, some papers have studied the formation of electrostatic complex, non-specific between charged polysaccharide and enzyme. The term "non-specific" is used to specify that this is not an enzyme-substrate complex and the active site of the enzyme is not involved.

For example, Lenormand, Deschrevel, Tranchepain, and Vincent (2008) studied the presence of electrostatic complex between hyaluronan and hyaluronidase by addition of proteins at different ionic strength and how they modulate hyaluronidase activity.

We have tested if the activity of pullulanase was disturbed by the negative charges of carboxymethylpullulan due to the presence of electrostatic interactions between the CMPull and pullulanase. We have observed a hydrolysis by increasing the concentration of salt in the medium (0.5 mol L^{-1}) to see the effect on pullulanase activity and to check if there are interactions between CMPull and pullulanase that prevent the enzyme to degrade the polymer.

Our results shown that enzymatic hydrolysis occurred normally and the reaction rate is almost the same as at 0.1 mol L^{-1} ionic strength. No electrostatic complexes between pullulanase and CMPull seem to occur and are not responsible for hydrolysis of CMPull.

3.4. Isothermal titration calorimetry

Calorimetry is a direct approach for studying the thermodynamics of enzyme function. ITC is particularly suited to measuring dynamic events such as binding and kinetics. ΔH_r is determined by integrating the total heat produced during catalysis of a known amount of substrate to product. Since the rate of heat production for a given amount of substrate is known, the rate of the reaction can be calculated (Jeoh, Baker, Ali, Himmel, & Adney, 2005). The reaction kinetic parameters can then be calculated from the

Table 2
Kinetics parameters for the hydrolysis of polysaccharides Pull, CMPulls with DS 0.16 and 0.8 respectively by pullulanase at 60 °C in $LiNO_3$ 0.1 mol L^{-1}.

	K_M (g L^{-1})	V_{max} (mol L^{-1} s^{-1})	K_{cat} (s^{-1})
Pull	7.0 (±0.1)	9×10^{-5} ($\pm 0.2 \times 10^{-5}$)	54 (±1)
CMPull 0.16	8.9 (±0.2)	1×10^{-5} ($\pm 0.1 \times 10^{-5}$)	6.6 (±0.2)
CMPull 0.8	14.4 (±0.3)	9×10^{-6} ($\pm 1 \times 10^{-6}$)	5.0 (±0.1)

Michaelis–Menten kinetics by using Eq. (4):

$$\vartheta = \frac{K_{cat}[E_{total}][S]}{K_M + [S]} \quad (4)$$

where K_{cat} is the turnover number of the enzyme, K_M (the Michaelis constant) is the substrate concentration at which the reaction velocity is half-maximal, $[E_{total}]$ is the total amount of enzyme present and [S] is the concentration of substrate.

The single injection method is a versatile approach that can be used to study both fast and slow kinetic reactions. In some cases, for example with self-hydrolytic or temperature unstable enzymes, or where catalysis is being conducted at an elevated temperature, it may be advantageous to add the enzyme into the substrate. ITC was used to study enzyme kinetics with the three substrates (Pull and both CMPulls) at 60 °C, pH 5.5 ($LiNO_3$ 0.1 mol L^{-1}). We observed the higher heat of enzymatic reaction for Pull. The lower heats for the two CMPulls are in good agreement with the difficulty to hydrolyze the modified substrates (Fig. 5). By converting raw heat rate to the catalytic rate (Fig. 6), we have calculated for each sample the maximal velocity (V_{max}) and the Michaelis constant (K_M) (Table 2). At first, the K_M and maximum velocity V_{max} were determined for pullulanase-Pull system. The values are coherent and not very different from those found in the literature. Our system evidences a K_M value of 7 g L^{-1} that can be compared to 1.33 g L^{-1} for *Clostridium* (Spreinat & Antranikian, 1990), 1.3 g L^{-1} for *Bacillus* sp. (Kunamneni & Singh, 2006), 5.4 g L^{-1} for *K. aerogenes* (Yamashita, Matsumoto, & Murooka, 1997), 5.76 g L^{-1} for *Thermococcus* (Erra-Pujada, Chang-Pi-Hin, Debeire, Duchiron, & O'Donohue, 2001) and the value increased to be 11 g L^{-1} for *amylopullulanase* (Lin, Chuang, Lin, 2008) and 16.4 g L^{-1} for *Alkaliphilic Bacillus* sp. (Lee, Lee, & Kim, 1997).

For the two CMPulls, we observed a clear decrease of V_{max} which is more pronounced when DS in carboxymethyl groups increases. The kinetics of enzymatic hydrolysis clearly decrease when the enzymatic activity is limited by the presence of carboxymethyl groups. In the same time, we observe an increase of K_M value, which is also directly proportional to carboxymethyl group number present among CMPull. K_M is an inverse measure of the affinity or strength of binding between enzyme and substrate. This shows that pullulanase had clearly some difficulties to bind to CMPull and that its affinity for the substituted substrate decreases when increasing the amount of substituted carboxymethyl group.

4. Conclusions

Pullulan and its derivatives were hydrolyzed by pullulanase and followed by both SEC/MALS/DRI characterization and reducing sugar determination. This approach appears to be appropriate for study and comparison of such hydrolysis. We have showed that ITC is a versatile technique to study enzyme kinetics of modified substrate. The enzymatic degradation occurs but the efficiency depends strongly on the substitution degree of polysaccharide. Enzymatic activity together with the kinetics of the reaction largely decreases when the DS of carboxymethyl groups increases. This can be related to the accessibility of enzyme to the substrate which is clearly limited by the presence of substituted groups.

Acknowledgments

We thank Dr. Vincent J.C. for his helpful discussion and his active support. We are grateful for funding from "Ministère de l'Education Nationale, de la Recherche et de la Technologie", Région Haute-Normandie and Réseau Polymères Innovants.

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