Carbohydrate Research 393 (2014) 60–69

Contents lists available at ScienceDirect

Carbohydrate Research

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

Colourimetric and fluorometric substrates for measurement of pullulanase activity

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ARTICLE INFO

Article history:
Received 14 February 2014
Received in revised form 16 April 2014
Accepted 18 April 2014
Available online 28 April 2014

Keywords:
Pullulanase
Limit dextrinase
Assay procedure
4,6-*O*-Benzylidene-2-chloro-4-nitrophenyl-β-maltotriosyl (1-6)-α-D-maltotrioside ($BzCNPG_3G_3$)
4,6-*O*-Benzylidene-methylumbelliferyl-β-maltotriosyl (1-6)-α-D-maltotrioside ($BzMUG_3G_3$)

ABSTRACT

Specific and highly sensitive colourimetric and fluorometric substrate mixtures have been prepared for the measurement of pullulanase and limit-dextrinase activity and assays employing these substrates have been developed. These mixtures comprise thermostable α- and β-glucosidases and either 4,6-*O*-benzylidene-2-chloro-4-nitrophenyl-β-maltotriosyl (1-6) α-maltotrioside ($BzCNPG_3G_3$, **1**) as a colourimetric substrate or 4,6-*O*-benzylidene-4-methylumbelliferyl-β-maltotriosyl (1-6) α-maltotrioside ($BzMUG_3G_3$, **2**) as a fluorometric substrate. Hydrolysis of substrates **1** and **2** by *exo*-acting enzymes such as amyloglucosidase, β-amylase and α-glucosidase is prevented by the presence of the 4,6-*O*-benzylidene group on the non-reducing end D-glucosyl residue. The substrates are not hydrolysed by any α-amylases studied, (including those from *Aspergillus niger* and porcine pancreas) and are resistant to hydrolysis by *Pseudomonas* sp. isoamylase. On hydrolysis by pullulanase, the 2-chloro-4-nitrophenyl-β-maltotrioside (**3**) or 4-methylumbelliferyl-β-maltotrioside (**4**) liberated is immediately hydrolysed to D-glucose and 2-chloro-4-nitrophenol or 4-methylumbelliferone. The reaction is terminated by the addition of a weak alkaline solution leading to the formation of phenolate ions in solution whose concentration can be determined using either spectrophotometric or fluorometric analysis. The assay procedure is simple to use, specific, accurate, robust and readily adapted to automation.

1. Introduction

Pullulanase (α-dextrin glucanohydrolase, pullulan 6-glucanohydrolase, limit dextrinase, amylopectin 6-glucanohydrolase)[1,2] (EC 3.2.1.4) cleaves the α-1,6-linkages in pullulan, amylopectin and α- and β-limit dextrins of starch. Hydrolysis of α-1,6-linkages in 1,4:1,6-α-gluco-oligosaccharides by pullulanase requires the presence of at least one α-1,4-linked glucosyl residue on each side of the α-1,6-linkage. Thus the smallest substrate for pullulanase is the tetrasaccharide 6^2-α-D-maltosyl maltose (**7**, Fig. 2).[3] Hydrolysis of glycogen is restricted by the highly branched, short chain length and dense nature of the molecule.[2,3] Several groups of enzymes catalysing *endo*-hydrolysis of pullulan have been described including those that hydrolyse the α-1,6-glucosidic bonds in pullulan and branched α-1,4;1,6 glucans (e.g., amylopectin)[1,4] (Type I) and those referred to as amylopullulanases[5] (Type II) which can hydrolyse both α-1,4- and α-1,6-glucosidic linkages. Other *endo*-acting enzymes active on pullulan include pullulan hydrolase type I (neopullulanase)[6] and type II (isopullulanase);[7] which only cleave α-1,4-linkages in pullulan releasing panose (**8**) or isopanose (**9**), respectively (Fig. 2). A fifth type of *endo*-hydrolase active on pullulan is referred to as pullulan hydrolase type III. This enzyme has the ability to hydrolyse both α-1,6- and α-1,4-glucosidic linkages in pullulan leading to the formation of panose (**8**) as well as maltose, maltotriose and glucose.[8]

Pullulanase finds widespread application in the starch processing industry. In combination with amyloglucosidase (AMG) it gives more efficient conversion of starch to glucose, reducing the formation of isomaltose (**10**, Fig. 2) and giving up to 1% greater yield of glucose.[9,10] High maltose syrups are produced from starch using combinations of pullulanase and β-amylase.[9,11]

The industrial application of pullulanase has led to significant interest in this enzyme and considerable screening efforts to locate new and interesting forms with different optimal conditions of pH and temperature for activity.

Pullulanase activity in industrial enzyme preparations can conveniently be assayed with reduced pullulan using an appropriate reducing sugar method such as that of Nelson[12] and Somogyi.[13] In crude enzyme preparations, the activity can be more specifically assayed using soluble dyed substrates (Red Pullulan) or dyed and cross-linked pullulan either in powder (AZCL-Pullulan) or tablet (Limit-Dextrazyme) form.[14] While the latter two substrates are

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http://dx.doi.org/10.1016/j.carres.2014.04.014
0008-6215/

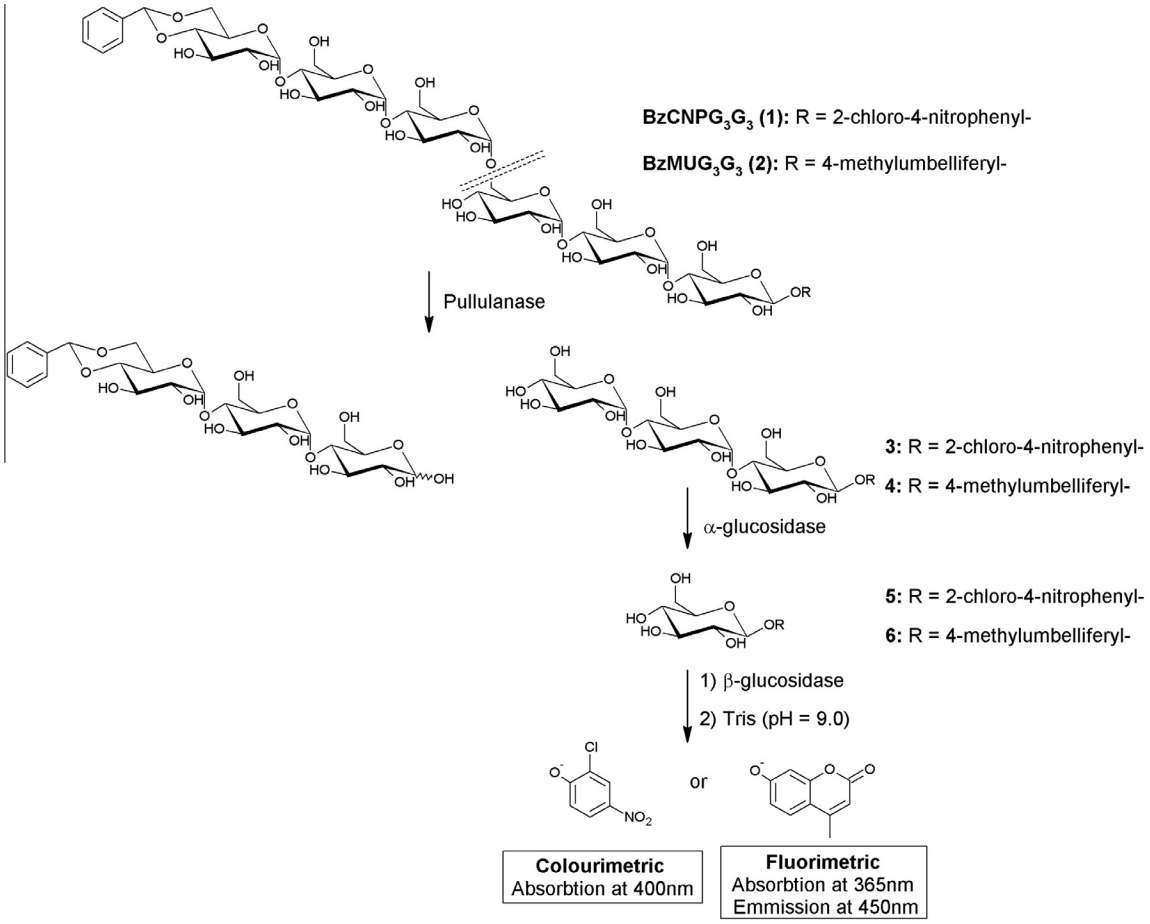

Figure 1. Overview of pullulanase assay procedure using $BzCNPG_3G_3$ (**1**) and $BzMUG_3G_3$ (**2**).

very useful and widely used, they do not readily lend themselves to automated analysis procedures.

The aim of this work was to develop simple assay procedures for pullulanase using the well-defined oligosaccharide, 6^3-α-D-maltotriosyl maltotriose (**11**, Fig. 2) based on similar procedures previously developed and published for the measurement of α-amylase[15,16] and *endo*-cellulase.[17] The two substrates developed were 4,6-*O*-benzylidene-2-chloro-4-nitrophenyl-β-6^3-α-D-maltotriosyl maltotrioside ($BzCNPG_3G_3$; **1**) and 4,6-*O*-benzylidene-4-methylumbelliferyl-β-6^3-α-D-maltotriosyl maltotrioside ($BzMUG_3G_3$; **2**). In this paper, the synthesis of these oligosaccharide substrates and their use in enzyme-linked assays for pullulanase will be described. During the course of this work the synthesis and use of the 2-chloro-4-nitrophenyl 6^3-α-D-glucosyl 6^3-α-D-maltotriosyl maltotrioside (**12**, Fig. 2) lacking a 4,6-*O*-benzylidene group for the assay of pullulanase and limit dextrinase were described.[18]

2. Results and discussion

6^3-α-D-Maltotriosyl maltotriose (**11**) was obtained in a highly pure form from Megazyme International. Oligosaccharide **11** is derived from pullulan which is composed mainly of repeating maltotriosyl units (~93%),[19] but also contains approximately 7% maltotetraosyl units. It is important that there is no contamination present in the 6^3-α-D-maltotriosyl maltotriose used for the synthesis of these substrates as the most common contaminant (heptasaccharide containing a maltotetraosyl unit) would be susceptible to hydrolysis by fungal α-amylases and thus would make the substrate less specific for the assay of pullulananse activity in enzyme mixtures.

The 2-chloro-4-nitrophenyl-(**1**) and 4-methylumbelliferyl-(**2**) substrates were prepared from 6^3-α-D-maltotriosyl maltotriose (**11**) following the procedure reported by Planas et al.[20] The 4,6-*O*-benzylidene protecting group was then introduced to prepare substrates **1** and **2** respectively as previously described for cello-oligosaccharides.[17] The principle of the assay procedures for pullulanase using **1** and **2** is shown in Figure 1. On hydrolysis of the oligosaccharide substrates **1** or **2** at the 1,6-α-linkage, the released 2-chloro-4-nitrophenyl-β-maltotrioside or 4-methylumbelliferyl-β-maltotrioside respectively are immediately hydrolysed to glucose and either 2-chloro-4-nitrophenol or 4-methylumbelliferone by the concerted action of the α-glucosidase and β-glucosidase enzymes in the reagent mixture. Reaction is terminated and phenolate ions are developed by addition of dilute alkali.

Unlike the substrate described by Bøjstrup et al.[18] these 4,6-*O*-benzylidene end-blocked substrates are absolutely resistant to all known *exo*-acting α-glucosidases and α-glucanases, such as AMG and β-amylase. Incubation of **1** under standard assay conditions with 200 Units of *Bacillus stearothermophilus* α-glucosidase, *Rhizopus* sp. AMG or barley β-amylase for up to 4 h gave no hydrolysis. In contrast, substrate **12** as used in the pullulanase assay procedure described by Bøjstrup et al.[18] is rapidly hydrolysed by AMG, and is even hydrolysed (*albeit* slowly) by the *B. stearothermophilis* α-glucosidase which is used in the reagent mixture. At a concentration of 1 mM and a pH of 5.5, the terminal non-reducing end 1,6-α-linked D-glucosyl residue in 6^3-α-D-glucosyl 6^3-α-D-maltotriosyl-maltotriose (**13**) is hydrolysed by *Bacillus stearothermophilis* α-glucosidase at 2% of the rate of hydrolysis of the non-reducing end 1,4-α-linked D-glucosyl residue in maltoheptaose. This observation introduces an instability issue into the substrate mixture