A GENERAL METHOD FOR DISTINGUISHING BETWEEN ENDO AND EXO ACTIONS OF CARBOHYDRASES

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1. Introduction

The use of hydrolytic enzymes of known specificities and action patterns has been, and continues to be, invaluable in the analysis of polysaccharide structures. Hydrolysis may proceed either by endo action, resulting in an essentially random hydrolysis of glycosidic linkages, or by exo action, in which the polysaccharides are degraded in a stepwise manner from the non-reducing ends of the polymeric chains. Several methods have been described for differentiating between the two action patterns but these generally suffer from the disadvantages that they may be used only for specific enzyme systems [1, 2] or they may require relatively large amounts of substrate and the isolation of hydrolytic intermediates [3]. This report describes a simple and sensitive method that requires only milligram quantities of substrate and does not involve the isolation of intermediate products. The method is of general application to depolymerases and its use is illustrated in the confirmation of the endo action pattern of pullulanase [2] with additional examples of endo and exo action pattern provided by α-amylase, β-amylase and *Aspergillus niger* amyloglucosidase.

2. Materials and methods

Pullulanase was prepared as by Wallenfels et al. [4] and *Aspergillus niger* amyloglucosidase as by Qureshi [5]. A twice crystallised preparation of sweet potato β-amylase was purchased from the Worthington Biochemical Corporation and a three times crystallised preparation of salivary α-amylase was prepared by the method of Fischer and Stein [6]. Glucose oxidase (Grade 11) and horseradish peroxidase (Grade 11) were obtained from the Boehringer-Mannheim Corporation, New York. Pullulan was prepared from *Pullularia pullulans* [7]. Amylose, average degree of polymerisation ($\overline{DP}$) 260, was purchased from Nutritional Biochemical Corporation and sodium metaperiodate from Sigma Chemical Company.

Total reducing sugars were determined as by Nelson [8] and glucose with glucose oxidase [9]. The concentrations of polysaccharide solutions were determined with phenol–sulphuric acid [10], or by acid hydrolysis [11] followed by estimation of reducing sugars or glucose. Enzymatic activities were measured as follows: α-amylase as by Robyt and Whelan [12], β-amylase as by Hobson et al. [13], amyloglucosidase by the release of glucose from soluble starch [5] and pullulanase by the method of Abdullah et al. [14].

Periodate oxidation of polysaccharides: Pullulan (25 mg) and amylose (10 mg) were each oxidised in 5 ml of solution at room temperature by the addition of sufficient 0.1 M sodium metaperiodate so that the molar ratio of glucose residues to periodate was 20 to 1. When all the periodate had been consumed (30 min), the digests were dialysed against water overnight. Solutions of unoxidised pullulan and amylose were similarly dialysed. After dialysis, portions (2.0 ml) of the oxidised and unoxidised pullulan solutions were treated overnight with sodium borohydride (4 mg). The solutions were then made 0.1 N in H_2SO_4, allowed to stand at room temperature for 18 hr, and neutralized to pH 7.0 with 0.5 N NaOH. Solutions (2 ml) of reduced and acid-treated pullulan (oxidized and unoxidized) and the unhydrolysed oxidised pullulan were chromatographed separately on Sephadex G-200 columns (fig. 1).

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3. Results and discussion

The method we are describing is an extension of the work of Nelson et al. [1] who made use of the special behaviour of laminarin, a 1→3-β-glucan, towards periodate. The oxidation can only take place at the chain ends and exo enzyme action is thereby blocked. It occurred to us that this same method is equally applicable to polysaccharides that can be oxidized at internal residues. Provided that oxidation is random, or at least is not confined to the reducing end, a slight degree of oxidation should protect most of the molecule from exo attack. Thus if pullulan, which has a degree of polymerization ($\overline{DP}$) of about 1450 [15], is randomly oxidized to the extent of 5%, it can be calculated that less than 1% of the molecule will remain unprotected from exo enzyme action.

That pullulan is randomly oxidized by periodate was proved by subjecting the oxidized polymer to a Smith degradation [16]. The oxidized pullulan was reduced and then treated at room temperature with dilute acid. Under these conditions hydrolysis occurs almost exclusively at the points of oxidation. The product was passed through Sephadex G-200 and the profile compared with those of oxidized pullulan, and unoxidized pullulan that had been reduced and treated with cold dilute acid. The latter two products were excluded from the gel (fig. 1) but the Smith-degraded pullulan was retarded and had clearly been randomly depolymerized. The experiment proved (a) that the oxidation was random and (b) that oxidation per se did not cause depolymerization.

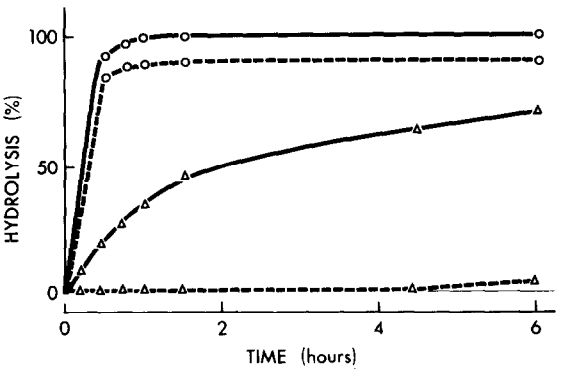

Fig. 2. Actions of pullulanase and amyloglucosidase on oxidized and unoxidized pullulan. Oxidized (- - - - -) or unoxidized (——) pullulan (1 mg/ml) was incubated at 30° with pullulanase (○, 0.5 IU/ml) or amyloglucosidase (△, 15 IU/ml) in digests containing 100 mM sodium acetate buffer, pH 5.0. Hydrolysis by pullulanase was measured by the release of reducing sugars and is expressed as % conversion into maltotriose. Hydrolysis by amyloglucosidase is expressed as % conversion into glucose measured with glucose oxidase.

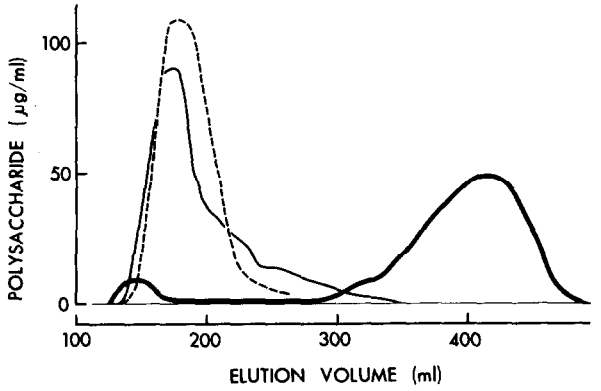

Fig. 1. Fractionation of the products of Smith degradation of pullulan. Solutions containing 10 mg of polysaccharide were passed through Sephadex G-200 columns (90 × 2.5 cm) in 10 mM sodium phosphate buffer, pH 6.0. The flow rate was 10 ml/hr and 5 ml fractions were collected. Carbohydrate was measured with phenol-sulphuric acid. Pretreatment of pullulan: periodate (- - - -); reduction, acid hydrolysis (——); periodate, reduction, acid hydrolysis (Smith degradation) (——).

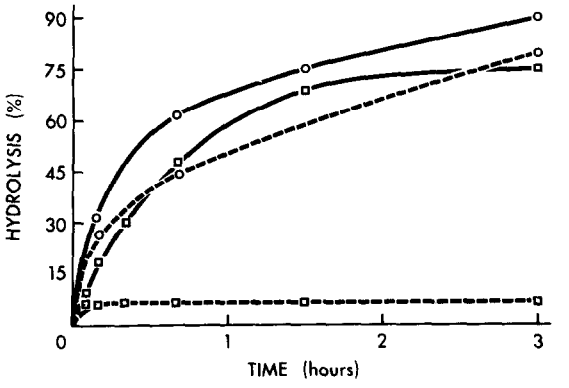

Fig. 3. Actions of α- and β-amylases on oxidized and unoxidized amylose. Oxidized (- - - -) or unoxidized (——) amylose (1 mg/ml) was incubated at 35° with α-amylase (○, 0.024 IU/ml) or β-amylase (□, 10 IU/ml). The α-amylase digests contained 10 mM NaCl and 50 mM sodium citrate buffer, pH 7.0, and the β-amylase digests contained 50 mM sodium acetate buffer, pH 5.0. Hydrolysis was measured by the release of reducing sugars and is expressed as % apparent conversion into maltose.

Fig. 2 depicts the actions of amyloglucosidase and pullulanase on oxidized and unoxidized pullulan. It is seen that while amyloglucosidase readily attacks pullulan, there is essentially no action on the oxidized polymer, a finding in keeping with the foregoing expectation concerning the behaviour of an exo-acting enzyme. By contrast, pullulanase attacked both the oxidized and unoxidized polymers. Moreover the addition of pullulanase to the digest of amyloglucosidase and oxidized pullulan resulted in an immediate release of glucose (not shown). This demonstrated that the amyloglucosidase was not subject to inhibition by the oxidized polysaccharide, but could attack unoxidized regions set free by pullulanase. The action pattern of the latter enzyme, previously claimed to be exo [17], is hereby confirmed as endo [2].

Oxidized amylose was used to demonstrate that the action patterns of α-amylase and β-amylase can be differentiated into endo and exo, respectively (fig. 3). A detectable hydrolysis (6%) was caused by β-amylase, in keeping with the lower molecular weight of the amylase ($\overline{DP}$ 260) as opposed to the pullulan ($\overline{DP}$ 1450).

The foregoing experiments demonstrate that the principle of using a periodate-oxidized polysaccharide to distinguish between exo and endo enzyme action can be applied generally, and not only to the special case where the polysaccharide is oxidizable only at its ends, as in the original demonstration of the principle by Nelson et al. [1]. Indeed a general method emerges for any type of polymer, e.g. protein or nuclei acid. All that is needed is a reagent that will randomly 'spatter' the polymer with groups that block exo enzyme action. This general applicability means that it is no longer necessary to design special methods appropriate only to the enzyme under test [e.g. 2]. Furthermore, the periodate method had the advantage of simplicity, requiring the measurement of only a single parameter, using milligram quantities of substrate. Other general methods [e.g. 3] have used as substrate a polysaccharide attackable by both an exo and an endo enzyme. In these methods it is necessary to make two measurements, comparing the number of bonds broken with the type of product formed.

Acknowledgements

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