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MEASUREMENT OF AMYLOGLUCOSIDASE USING P-NITROPHENYL ß-MALTOSIDE AS SUBSTRATE

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SUMMARY

An enzyme-linked assay for the measurement of amyloglucosidase in commercial enzyme mixtures and crude culture filtrates is described. A method for the synthesis of the substrate employed, *p*-nitrophenyl ß-D-maltoside, is also described. The substrate is used in the presence of saturating levels of ß-glucosidase. With a range of *Aspergillus* sp. culture filtrates, an excellent correlation was found for values obtained with this assay and a conventional assay employing maltose as substrate with measurement of released glucose.

INTRODUCTION

Amyloglucosidase (glucoamylase; EC 3.2.1.3) finds widespread industrial application in the conversion of starch and malto-oligosaccharides to glucose (Pazur and Ando,1959). Enzymes have been purified and characterised from a wide range of micro-organisms and the action patterns on a range of linear and branched maltodextrins studied in detail. Amyloglucosidase has been assayed using maltodextrins, starches and starch fractions. In industrial microbial preparations of amyloglucosidase, high and varying levels of α-amylase and transglucosidase (α-glucosidase)(McCleary *et al*, 1989) are also usually present. For this reason, the preferred substrate is maltose, as this is not hydrolysed by α-amylase. The rate of hydrolysis of this substrate has been measured by reducing sugar methods (Somogyi, 1952) and by the measurement of released glucose using glucose oxidase-peroxidase reagent (Lloyd and Whelan, 1969) or a hexokinase-glucose-6-phosphate dehydrogenase coupled assay (Bergmeyer *et al*, 1974). Maltose is attacked by transglucosidase, but this enzyme is generally only a minor contaminant in culture filtrates and is usually removed from industrial enzyme mixtures by adsorption procedures (McCleary *et al*, 1989). A continuous spectrophotometric assay for amyloglucosidase has been published, (Sabin and Wasserman, 1987) but the assay employs maltotetraose as substrate and this oligosaccharide is rapidly hydrolysed by *Rhizopus* and *Aspergillus sp.* α-amylases.

In the current paper we describe a procedure for the synthesis of *p*-nitrophenyl ß-maltoside and for the use of this substrate in a simple, rapid and specific assay for the measurement of amyloglucosidase in crude or purified enzyme preparations. The assay is based on the use of *p*-nitrophenyl ß-maltoside in the presence of saturating levels of ß-glucosidase; when the terminal α-linked D-glucosyl residue is removed by amyloglucosidase, the ß-glucosidase gives immediate removal of the ß-linked D-glucosyl residue, with release of free *p*-nitrophenol, which is measured spectrophotometrically.

MATERIALS AND METHODS

Materials

All chemicals employed were of the highest purity commercially available.
Microbial culture broths were provided by Biocon Biochemicals, Carrigaline, Co. Cork, Ireland. Pure *Aspergillus niger* amyloglucosidase, α-amylase and transglucosidase were prepared by Megazyme Pty. Ltd. Yeast maltase was obtained from Genzyme Biochemicals, Maidstone, Kent, U.K. and almond emulsin ß-glucosidase from Seppim, Chemin de la Chalerie, Sees, France.

Methods

Synthesis of *p*-nitrophenyl ß-maltoside

(a) Synthesis of acetylated *p*-nitrophenyl ß-maltoside

Method 1: To a solution of 2,3,6,2',3',4',6'-tetra-*O*-acetyl-α-maltosyl bromide (Brauns, 1929), freshly prepared from octa-*O*-acetyl-ß-maltose (10 g, 14.8 mmol), in dichloromethane (20 ml distilled over phosphorus pentoxide) and 2-propanol (80 ml), *p*-nitrophenoxide bound to Amberlyst A-26 prepared as described (Iversen and Johansson, 1979)(20 g) was added. The suspension was shaken for 72 h at room temperature, diluted with dichloromethane, and filtered. The resin was washed with dichloromethane, and the combined organic phases were washed with water, dried, evaporated and purified by flash-chromatography on silica gel with light petroleum-ethyl acetate (1.5 : 1 v/v). The expected compound was crystallised from ethanol (2.6 g, 24 %), m.p. 176-177°, $[\alpha]_D$+ 33.5 (**c** 1.0, chloroform); m.p. 175-176°; $[\alpha]_D$ + 33.8 (**c** 1.4, chloroform). ^{13}C-N.m.r. data ($CDCl_3$); ∂ (p.p.m.) 97.5 (C-1); 95.7 (C-1'); ^{1}H N.m.r. data ($CDCl_3$): ∂ (p.p.m.) 5.37 (H-1'); 5.30 (H-3'); 5.27 (H-3); 5.20 (H-1); 5.06 (H-2); 4.96 (H-4'); 4.80 (H-2'); 4.43 (H-6'a); 4.2-3.8 (H-6'b, H-5, H-4, H-6a, H-6b, H-5').

Anal calc. for $C_{32}H_{39}O_{20}N$: C, 50.74; H, 5.19; N,1.85. Found : C, 50.81; H, 5.28 and N, 1.86 %.

Method 2: To a solution of maltosyl bromide, obtained as described above, in acetonitrile (30 ml), potassium *p*-nitrophenoxide (2.9 g, 16.3 mmol, prepared according to Kleine and Sidhu, 1988) and dicyclohexyl-18-crown-6 (0.52 g, 1.4 mmol) were added. After 48 h at room temperature, the mixture was filtered and the organic phase evaporated. The reaction mixture was then diluted with dichloromethane, washed with cold water, dried and purified as above. The expected compound was obtained in 14 % yield after crystallisation.

(b) Synthesis of *p*- nitrophenyl ß-maltoside

The acetylated compound (2.5 g, 3.3 mmol) was deacetylated by treatment with sodium methoxide (**M**, 1 ml) in methanol (80 ml). After neutralisation and freeze drying, *p*-nitrophenyl ß-maltoside was obtained (1.5 g, 100 %)$[\alpha]_D$ + 7.0 (**c** 1.1, methanol); this compares to a value in literature (Babers and Goebel, 1934) of $[\alpha]_D$ + 6.0 (**c**, methanol). ^{13}C N.m.r. data (CH_3OD): ∂ (p.p.m.) 103.8 (C-1); 102.4(C-1').

Anal. calc. for $C_{18}H_{25}O_{13}N$. 0.5 H_2O: C, 45.76; H, 5.55; N, 2.96. Found : C, 46.65; H, 5.44 and N 3.02 %.

The absence of the α-anomer (i.e. *p*-nitrophenyl α-maltoside) was confirmed by treatment of the substrate (in the absence of ß-glucosidase) with maltase or amyloglucosidase (2 Units). There was no detectible release of free *p*-nitrophenol.

Synthesis of *o*-nitrophenyl- and *m*-nitrophenyl ß-maltosides

These compounds were prepared by essentially the same procedure as that for *p*-nitrophenyl ß-maltoside except that *o*-, and *m*-nitrophenoxides were employed.

Preparation of nitrophenyl ß-maltoside/ ß-glucosidase mixtures

ß-Glucosidase (12.1 U/mg) was dissolved in distilled water to give a final concentration of 200 U/ml. The nitrophenyl ß-maltosaccharides were prepared at a concentration of 20 mM in distilled water. These components were mixed to give the desired range in concentration of substrate and ß-glucosidase. A ready-to-use reagent is available from Megazyme Pty. Ltd.

Assay of amyloglucosidase

Pre-equilibrated and suitably diluted amyloglucosidase solution (0.2 ml) in 100 mM sodium acetate buffer (pH 4.5) was added to 0.2 ml of pre-equilibrated substrate mixture (unbuffered) and incubated at 40° C for exactly 10 min. The reaction was terminated and colour developed by the addition of Trizma base (3.0 ml, 1 % w/v, pH > 10), and the absorption at 410 nm measured (E_{mM} of *p*-nitrophenol at pH > 10 is 17.8). One unit (U) of enzyme activity is defined as the amount of enzyme required to release 1 µmole of *p*-nitrophenol/min under the defined assay conditions.

Calculation of activity:

Amyloglucosidase (U/ml or U/g) ;

$$= \frac{\Delta A_{410nm}}{\text{Incubation time}} \times \frac{\text{Total volume in assay cell}}{\text{Aliquot assayed}} \times \frac{1}{E_{mM}} \times \text{Dilution}$$

$$= \frac{\Delta A_{410nm}}{10} \times \frac{3.4}{0.2} \times \frac{1}{17.8} \times \text{Dilution}$$

RESULTS AND DISCUSSION

Synthesis of *p*-nitrophenyl ß-maltoside

The first and only report of the preparation of this compound was by Babers and Goebel (1934), wherein they reported a yield of 20 % from acetobromomaltose. In our hands, attempts to increase the yield using conventional methods for the synthesis of 1,2-*trans*-glycosides failed. Only two procedures led to the synthesis of the expected product with a similar yield to that already reported. The first method combined the advantage of solid-phase synthesis and the activation of *p*-nitrophenol using *p*-nitrophenoxide bound to an ion exchange resin. This method was slightly different to the one described for the synthesis of nitrophenyl ß-D-gluco- and galactopyranosides (Iversen and Johansson, 1979). Acetylated *p*-nitrophenyl ß-maltoside was isolated in 24 % yield after crystallisation. The assignment of the anomeric configuration of the aglycone was clearly inferred from an examination of its n.m.r. spectra. The ^{13}C n.m.r. spectrum shows for C-1 a chemical displacement at 97.5 p.p.m. for the ß-D-glucopyranosyl residue. Its α-anomer, which was also obtained (3 %) yield, shows a chemical shift for C-1 at 94.1 p.p.m.. In the ^{1}H-n.m.r. spectrum, the coupling constant for H-1--H-2 of 7.8 HZ is a further confirmation of the ß-D-anomeric configuration of the aglycone. The second method which was used is based on the *SN2* reaction of the potassium salt of *p*-nitrophenol with acetobromomaltose in the presence of catalytic amounts of dicyclohexyl-18-crown-6. In this case the expected compound was isolated in 14 % yield. Subsequent de-*O*-acetylation of the acetylated compond gave *p*-nitrophenyl ß-D-maltoside in quantative yield.

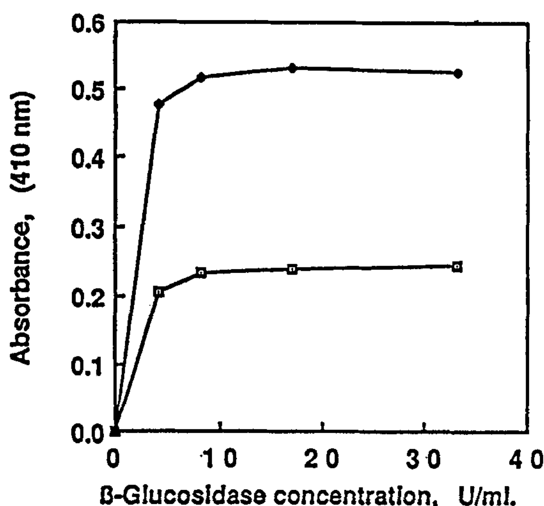
Optimisation of the Amyloglucosidase assay reagent

The effect of the concentration of ß-glucosidase on the sensitivity of the assay under otherwise optimal conditions (at a *p*-nitrophenyl ß-maltoside concentration of 2 mM in the final assay mixture) is shown in Figure 1. It is apparent that the enzyme is at saturating concentration at 15 U/ml. In all subsequent experiments, a concentration of 25 U/ml was employed.

In the assay of amyloglucosidase using maltose as substrate, there is a potential overestimation due to the presence of transglucosidase in the enzyme preparation and the action of this on maltose. In the current study, it has been found that transglucosidase also acts on *p*-nitrophenyl ß-maltoside, and that the relative rates of hydrolysis of maltose and *p*-nitrophenyl ß-maltoside by each of the enzymes is essentially the same.

In attempts to improve the specificity of the assay for the measurement of amyloglucosidase, the *m*- and *o*-nitrophenyl ß-maltoside derivatives were prepared and evaluated. In Figure 2, the effect of substrate type and concentration on the assay of amyloglucosidase is shown. The *p*-nitrophenyl derivative is the substrate of choice, giving a greater sensitivity than either *m*-,

or *o*-nitrophenyl ß-maltoside. Since, the rates of cleavage of each of these substrates relative to maltose, by amyloglucosidase and transglucosidase, were essentially the same, then, the *p*-nitrophenyl derivative is the substrate of choice. This is used at a concentration of 2 mM in the final assay mixture.



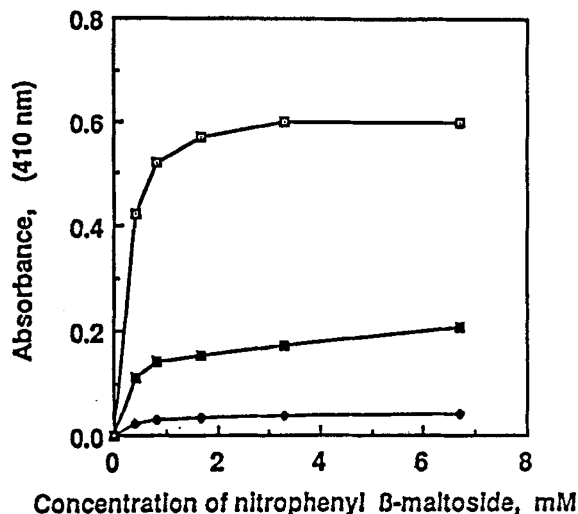


Fig. 1. Optimisation of ß-glucosidase concentration in the amyloglucosidase assay reagent. Incubation time: □ , 5 min; ◆,10 min.

Fig. 2. Optimisation of the concentration of *p*-nitrophenyl ß-maltoside in the reagent mixture. Substrates: ■,*o*-; ◆ ,*m*-; □ ,*p*-nitrophenyl ß-maltosides.

In a comparison of values obtained for amyloglucosidase activity (Units/ml or gram) in a range of *Aspergillus* sp. enzyme preparations, a ratio of 1.8 for activity on maltose / activity on p-nitropheny ß-maltoside was routinely obtained.

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