0273-2289/97/6601-0025$9.50

Synthesis of Isopropyl-1-Thio-β-D-Glucopyranoside (IPTGlc), an Inducer of *Aspergillus niger* B1 β-Glucosidase Production

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Received November 21, 1995; Accepted January 5, 1996

ABSTRACT

Production of β-glucosidase in *Aspergillus niger* B1 is subjected to catabolic repression by glucose. *Aspergillus niger* B1 grown on bran as a carbon source secreted β-glucosidase. The maximum level of the enzyme was reached after 7 d of fermentation. Addition of 1% glucose to the medium suppressed β-glucosidase production to undetectable levels. In this study, the organic synthesis of a potential inducer of β-glucosidase production by *A. niger* B1's reported. Isopropyl-1-thio-β-D-glucopyranoside (IPTGlc) was synthesized using a two-step organic synthesis protocol. The H-NMR data agreed with those reported previously for the galactoside analog. When IPTGlc was added 24 h after inoculation at a final concentration of 0.4 m*M*, similar levels of β-glucosidase were reached 3 to 4 d earlier as compared to fermentation without IPTGlc induction. In practice, this may translate to a more efficient method of producing β-glucosidase from this fungus.

Index Entries: *Aspergillus niger*; β-glucosidase; isopropyl-1-thio-β-D-glucopyranoside; induction.

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INTRODUCTION

β-glucosidases are currently intensively produced for scientific as well as industrial purposes *(1–3)*. *Aspergillus* species are known as a useful source of β-glucosidases *(4–6)*. *Aspergillus niger* is by far the most efficient producer of β-glucosidase among investigated micro-organisms *(4)*. *Aspergillus* β-glucosidase is less specific to the substrate, stable at higher temperatures, and active at pHs lower than that from other fungal sources *(5)*. Another advantage of *A. niger* is that it is considered nontoxic and can, therefore, be used in the food industry *(7)*.

Shoseyov et al. *(8)* have described a unique β-glucosidase from *A. niger* B1, which is active at low pHs as well as in the presence of high ethanol concentrations. This enzyme effectively hydrolyzes flavor-compound glycosides in certain low-pH products, such as wine and passion-fruit juice, thereby enhancing their flavor *(9)*. *Aspergillus niger* β-glucosidase production is affected by carbon source *(10)*. When bran was used as the carbon source, the *A. niger* B1 β-glucosidase was secreted into the medium in the late stationary phase of fungal growth. At this stage, much of the mycelium has lysed and protease activity is elevated in the medium *(11)*.

Production of β-glucosidase in many micro-organisms is susceptible to catabolic suppression *(12–14)*, with several inducers having been reported. Sophorose induces β-glucosidase production in *Trichoderma viride (14)*. Cellulose, cellobiose, and carboxymethylcellulose induce β-glucosidase production in *Myceliophthora thermophila (15)*. In this organism, the addition of *p*-nitrophenyl β-D-glucopyranoside to a cellulose medium increased the production of β-glucosidase fivefold.

Thio-analogs of O-glycosides, whereas being poor substrates for glycosides are excellent inducers. Isopropyl-thio-β-galactoside (IPTG) is an efficient inducer of the lactose operon in *E. coli*. It is widely used to induce expression of recombinant proteins *(16)*. This β-thio-galactoside is relatively stable as compared to the O-galactoside.

Here the two-step organic synthesis of isopropyl-1-thio-β-D-glucopyranoside (IPTGlc), and its ability to induce production of β-glucosidase in *A. niger* B1 is reported.

MATERIALS AND METHODS

Synthesis of Isopropyl-Thio-β-D-Glucopyranoside (IPTGlc)

Synthesis of IPTGlc (glucoside) was performed essentially according to the protocol for the synthesis of the galactoside analog by Carlsson et al. *(17)*.

Synthesis of Isopropyl-2,3,4,6-Tetra-O-Acetyl-1-Thio-β-D-Glucopyranoside

Stannous chloride (0.3 mL, 25 mmol) was dripped into a stirred solution of 1,2,3,4,6,-penta-*O*-acetyl-β-D-glucopyranose (15 g, 38.5 mmol) and 2-propanethiol (7.3 mL, 77 mmol) in dichloromethane (200 mL) containing

5 g powdered molecular sieves (4Å), held at –40°C using dry ice for 8 h. Solid sodium hydrogen carbonate (5 g) was then added to the solution. The mixture was allowed to reach 5–10°C, then filtered through a pad of celite 545. An aqueous solution of sodium hydrogen carbonate (8% w/v) was carefully added to neutralize the solution. The organic phase was separated in a separatory funnel, and washed with 100 mL aqueous sodium hydrogen carbonate and 100 mL water. It was then dried over $MgSO_4$, filtered and concentrated. The product was analyzed by TLC (silica gel 60, Merck, Mannheim, Germany), using toluene:ethyl acetate (2:1). Isopropyl-2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside was detected at Rf = 0.55, as were trace amounts of the starting material. The yield of isopropyl-2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside was 10.6 g (75%), obtained as a colorless oil.

H-NMR data: ($CDCl_3$, TMS):d 1.31 (d, 6H, $CH(\underline{CH_3})_2$:2.01, 2.03, 2.05, 2.07 (4_S, 12H, $4CH_3$); 3.17 (m, 1H, $\underline{CH}$ $(CH_3)_2$), 3.65–4.3 (m, 3H, H-5, H-6_{ab}); 4.59 (d, 1H, H-1); 5.00–5.26 (3d, 3H, H-2,3,4).

Synthesis of Isopropyl-1-Thio-β-D-Glucopyranoside (IPTGlc)

Sodium methoxide was prepared by addition of 230 mg sodium to 10 mL dry methanol. The reagent was added to the 10.6 g of isopropyl-2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside in 90 mL methanol. The solution was stirred at RT overnight and neutralized by adding ion-exchanger (Dowex-H^+). TLC analysis using CH_2Cl_2:methanol (4:1) confirmed the presence of IPTGlc at Rf = 0.62 as the major product. The solution was filtered and concentrated at reduced pressure to give a thick, syrupy residue (6.65 g, 77%). H-NMR data: (D_2O):d 1.32 (d, 6H, CH $(\underline{CH_3})_2$); 3.2 (m, 1H, $\underline{CH}$ $(CH_3)_2$); 3.4–3.63 (m, 5H, H-2, H-3, H-5, H-6ab); 3.87 (m, 1H, H-4) 4.54 (d, 1H, H-1).

Organism, Media, and Culture Conditions

Aspergillus niger B1 (CMI CC 324626) was obtained as previously described *(8)* and cultured, with some modifications. The *A. niger* B1 was grown at 35°C for 7 d in an orbital shaker (250 rpm) in 250 mL flasks containing 1% (w/v) bran with or without 1% (w/v) glucose in $(NH)_2SO_4$ $3H_2O$ (0.5 g/L), KH_2PO_4 (0.2 g/L), $MgSO_4$ (0.2 g/L), $CaCl_2H_2O$ (0.1 g/L), $FeSO_4$ $6H_2O$ (0.001 g/L), $ZnSO_4$ $7H_2O$ (0.001 g/L), and 2 m*M* citric acid, at pH 3.4. The medium was autoclaved, cooled, inoculated with *A. niger* B1 (10^6 spores/mL), and fermented for 7 d. IPTGlc at a final concentration of 0.4 m*M* was added 24 h after inoculation. Each day a 1-mL sample of the fermentation broth was taken under sterile conditions, and immediately frozen at –20°C.

Enzyme Assay

A. niger B1 β-glucosidase was obtained from filtered culture medium as previously described *(8)*. β-glucosidase activity was determined by the hydrolysis of 4-methylumbelliferyl-β-D-glucoside (MUG) and the release

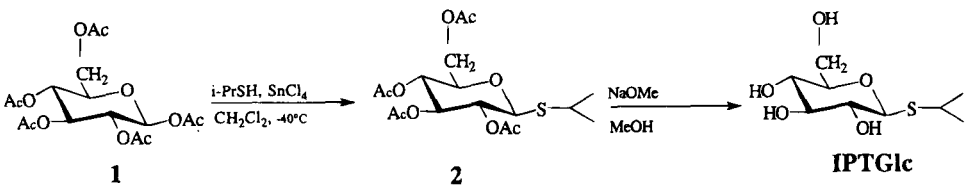

Fig. 1. Steps in the organic synthesis of isopropyl-1-thio-β-D-glucopyranoside (IPTGlc). 1. 1,2,3,4,6,-penta-*O*-acetyl-β-D-glucopyranose. 2. Isopropyl-2,3,4,6,-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside.

of fluorescent 4-methylumbelliferone (MU) *(18)*. The fluorescence of MU was determined with a fluorometer; 1 U of β-glucosidase was defined as the formation of 1 μmol MU/min.

RESULTS AND DISCUSSION

The two-step organic synthesis protocol used to synthesize IPTGlc is summarized in Fig. 1. H-NMR data agreed with those reported previously for the galactoside analog *(17)*. The high coupling constant (10.1 Hz) of the anomeric proton confirmed the β conformation of the glucoside.

A. niger grown on bran as a carbon source secreted β-glucosidase. The maximum level of the enzyme was reached after 7 d of fermentation (Fig. 2). Addition of 1% glucose to the medium suppressed β-glucosidase production to undetectable levels (Fig. 2). Suppression of β-glucosidase production by glucose, has already been reported in *Trichoderma harzianum (19), Trichoderma viride (20,21)*, a *Streptomyces* sp. *(22)*, and *Myceliophthora thermophila (15)*. Glucose concentrations higher than 0.05% caused suppression of β-glucosidase production in *Aspergillus niger* VKMF-1092 *(13)*. Addition of 0.4 m*M* IPTGlc, 24 h after inoculation induced early production of β-glucosidase in *A. niger* B1 significantly (Fig. 2), as determined by Duncan's multiple range test ($p \leq 0.05$). Higher levels of IPTGlc (4 m*M*) or repeated addition of the IPTGlc 24 and 72 h after inoculation did not significantly alter the effect of a single addition of IPTGlc 24 h after inoculation (not shown).

These results demonstrate that the process of β-glucosidase production in *A. niger* B1 could be shortened by 3–4 d by the addition of IPTGlc as compared to the fermentation without IPTGlc induction (Fig. 2). This indicates the possibility to design a more efficient method to produce β-glucosidase from this and related fungi. The usefulness of IPTGlc in other organisms requires additional investigation. The existing knowledge of β-glucosidase regulation in different micro-organisms is fragmentary and controversial. It was established that the use of different carbohydrates substrates in the medium or addition of inducers can affect the level of suppression of cellulase production in *Pseudomonas fluoresencs*, however, there was no effect on β-glucosidase, an intracellular enzyme in this organ-

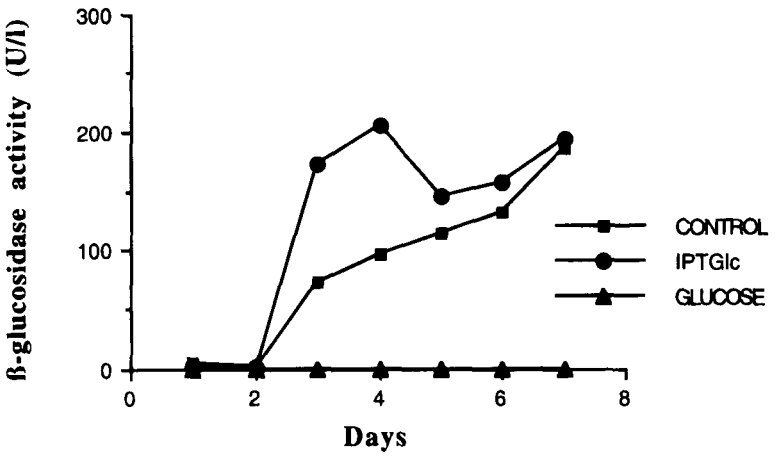

Fig. 2. Production of β-glucosidase by *A. niger* as affected by glucose and isopropyl-1-thio-β-D-glucopyranoside (IPTGlc).

ism *(23)*. Production of β-glucosidase by *T. viridae (14)* and *A. phoenicis (12)* seems to be constitutive, although it is influenced by the composition of growth medium. Some media were shown to enhance β-glucosidase production. However, the contribution of each medium component could not be characterized definitely *(6)*. Use of defined media and a defined inducer such as IPTGlc could contribute significantly to the understanding of β-glucosidase regulation in various industrial organisms.

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