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A RAPID CHITIN SYNTHASE PREPARATION FOR THE ASSAY OF POTENTIAL FUNGICIDES AND INSECTICIDES

D.J. Adams* and G.W. Gooday

Department of Microbiology,
University of Aberdeen,
Marischal College,
Aberdeen AB9 1AS

SUMMARY

A rapid technique for the preparation of large quantities of solubilized chitin synthase is described. The enzyme derived is very stable and has high specific and total activities. It has been used to screen compounds with potential fungicidal and insecticidal properties.

INTRODUCTION

Chitin is found in fungi and arthropods but not in green plants or mammals. Thus considerable interest has been shown in chitin synthase as a potential target for compounds with fungicidal or insecticidal activity. In order to assess the usefulness of such compounds, large quantities of stable, solubilized enzyme are required. The stipes of the mushroom Coprinus cinereus provide the most prolific known source of chitin synthase in terms of specific activity of enzyme in the initial extract (Gooday, 1979). The preparation of solubilized enzyme has however involved a time-consuming procedure. We describe a rapid extraction technique producing a stable, solubilized enzyme preparation of very high specific activity.

MATERIALS AND METHODS

Media and Culture. Coprinus cinereus (dikaryon of 535/01 x 177/01 from Dr. R.F.O. Kemp, University of Edinburgh) was maintained on slopes of malt agar (2% Oxoid agar no. 3, 1% Oxoid malt extract). For fruiting, a piece of mycelium was transferred from a maintenance culture to the centre of 100 ml of fruiting medium (2% Oxoid agar no. 3, 1% Oxoid malt extract, 0.4% Oxoid yeast extract and 0.4% glucose) in 500 ml wide-necked conical flasks stoppered with polyurethane bungs. Flasks were incubated at 37°C for four days in the dark. They were then incubated at 25°C for two to three days with a cycle of 16 hours light and 8 hours dark. Fruit bodies of approximately 2 cm in length were harvested and the stipes separated from caps and adhering mycelium.

Tissue disruption. Stipes were disrupted using a 25 ml X-Press with 20 ml disruption buffer (0.02 M-Tris-HCl pH 7.5, 0.001 M-EDTA$(Na)_2$, 0.01 M-Mg$(Cl)_2$, 0.5 M-NaCl; containing 200 mg of digitonin (Koch-Light Laboratories) dissolved by briefly heating the solution to 100°C). The press was cooled to -20°C and approximately 2 ml of the disruption buffer was added. This froze instantly and plugged the central pore of the apparatus. Stipes (5 g) were added and the remainder of the disruption buffer poured over them. The apparatus was then re-cooled to -20°C for a period varying from 3 h to overnight (yield was not affected by this period of storage). The tissue was disrupted by subjecting the apparatus to a pressure of 200 MPa in a hydraulic press. The pressure cell was inverted and the procedure repeated.

Centrifugation. The homogenate was recovered from the pressure cell, thawed and centrifuged at 35000 g (r_{av} 80 mm) for 30 min at 4°C in a Sorvall RC-5 Superspeed centrifuge using an SS-34 rotor. The supernatant was stored at -20°C as the solubilized enzyme preparation.

That the enzyme was composed entirely of "solubilized" enzyme and did not contain "particulate" membrane-bound enzyme activity, was ascertained by applying the criterion of

centrifugation of a sample at 100000 g (r_{av} 73mm) for 60 min in a Beckman L2-5B ultracentrifuge using an SW 50.1 rotor. All of the enzyme activity was recovered in the supernatant.

Assay for chitin synthase and for protein. The reaction mixture (50 µl) contained 10 µl of enzyme preparation and final concentrations of 50 mM-Tris-HCl pH 7.5, 10 mM-$Mg(Cl)_2$, 1 mM-$EDTA(Na)_2$ and 0.6 mM-uridine diphospho-N-acetylglucosamine containing 25 nCi of UDP-[U-^{14}C]-N-acetylglucosamine (323 mCi/mmol; Radiochemical Centre, Amersham). Incubations were for 30 min at 25°C in 1.5 ml plastic reaction tubes, and the reaction was stopped by incubating for a further 2 min at 100°C. The entire contents of the tube were filtered through an Amicon microporous filter (pore size 0.2 µm) which had been pre-washed in 20 mM-$Na_4P_2O_7$ to prevent any unreacted UDP-GlCNAc from sticking to the filter. The tube was washed twice with 50µl of 1% (v/v) aqueous Triton X-100 and the washings filtered. The filter was finally washed with 15 ml distilled water, dried at 70°C for 20 min and placed in 4 ml scintillation fluid containing 4 g 2,5-diphenyloxazole (PPO) and 0.1 g 1,4-di-2-(5-phenyloxazolyl)-benzene (POPOP) per litre of toluene. Radioactivity was counted in a Packard Tricarb liquid scintillation counter. (This procedure is specifically designed for rapid, accurate and reproducible assays of these preparations, but is not suitable for detailed kinetic studies).

Protein was determined by the method of Lowry et al., (1951); using bovine serum albumin (dissolved in disruption buffer) as standard.

RESULTS AND DISCUSSION

This procedure results in the extraction of approximately 20 ml of solubilized enzyme preparation. This is more than five times the amount derived from the previously described technique (Gooday and de Rousset-Hall, 1975). Only one hour is required per preparation, with no requirement for ultracentrifugation. Enzyme is derived of specific activity comparable with that prepared by the previous method. For example, consecutive preparations had specific activities of 20 and 23 nmol GlcNAc

incoporated min^{-1} mg $protein^{-1}$. In terms of total activity per ml, this preparation gives yields as much as an order of magnitude higher than those derived from the original preparative procedure. The enzyme is particularly stable, with negligible loss of activity over a storage period of several months at $-20^{o}C$.

Thus, potential inhibitors of chitin synthase can be screened readily against the solubilized enzyme so that the described system is ideal for the assay of potential fungicides and insecticides. For example, it has been used successfully for a study of the inhibition of the enzyme by a range of analogues of the substrate, UDP-GlcNAc(D.J. Adams and G.W. Gooday, unpublished results).

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