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A VERSATILE ASSAY FOR TOTAL CELLULASE ACTIVITY USING U-[^{14}C]-LABELLED BACTERIAL CELLULOSE

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SUMMARY

A versatile and sensitive assay for total cellulase activity and not just carboxymethylcellulase activity, is described. The method is equally suitable for studying the kinetics of solubilization of cellulose by growing cells or isolated enzyme fractions.

INTRODUCTION

The most commonly used methods for measuring either total cellulolytic activity or the activity of single enzyme components of cellulase complexes are based on monitoring the rate of release of reducing sugars from a cellulose substrate of choice. A limitation of these methods is that they determine reducing end groups irrespective of the size of the soluble products of which these form part. Therefore, similar extents of accumulation of reducing sugars by cellulase complexes do not necessarily indicate comparable extents of solubilization. Also, where the role of different carbohydrates in the regulation of cellulases is to be studied, the production of reducing sugars has to be measured against the background of the carbohydrates added to the incubation mixtures, which reduces the sensitivity of the assays. Furthermore, these methods are not applicable to the study of the influence of environmental factors such as pH on the rate of cellulose degradation by growing microbial cultures because, in this case, the soluble sugars are generally metabolized as rapidly as they are produced by the cellulases.

The present communication describes a simple, versatile method which overcomes these limitations. The time course of release of radioactivity from U-[^{14}C]-labelled insoluble cellulose is followed by scintillation counts on supernatant fractions from either growing

cultures or enzyme incubation mixtures. The substrate used is Acetobacter cellulose which is a native (type 1) cellulose with a degree of polymerization of >3000 (Hestrin, 1963), a crystallinity index considerably higher than that of the much-used substrates filter paper and Avicel, and a high total pore volume (Grethlein, 1985). Despite its higher crystallinity, this substrate is more readily degraded by a larger section of fibrolytic bacterial species than filter paper or Avicel. These properties, together with the ease with which this cellulose can be radiolabelled to a desired specific activity make it a highly suitable substrate for studies of the kinetics of cellulose solubilization. The utility of the method is illustrated by results of studies on crude and purified enzyme fractions from Trichoderma reesei and on growing cultures of a strain of the rumen anaerobe Ruminococcus flavefaciens.

MATERIALS AND METHODS

Organisms. Ruminococcus flavefaciens strain FD1 was a gift from Prof. M P Bryant of the University of Illinois, Urbana-Champaign, while Acetobacter xylinum strain ATCC 23770 was obtained from the American Type Culture Collection.

Preparation of [U-^{14}C]-labelled bacterial cellulose. To a medium containing 0.5% (w/v), each, of yeast extract and peptone, and 1.0% (w/v) glucose monohydrate in potassium phosphate buffer pH 6.3, sufficient D-[U-^{14}C]-glucose was added to give a specific activity of approximately 0.8 µCi/mmole glucose. The medium was dispensed in 500 ml volumes into large Roux flasks, giving a layer depth of about 10 mm. The flasks were inoculated with 10 ml volumes of the liquid phase of 2 week old cultures of A. xylinum in which the glucose had been depleted, and incubated at 30°C for two weeks. A cellulose pellicle forms at the liquid surface and can be removed as a single sheet. On average, dry weight yields were 1 g/l cellulose. To remove adhering culture medium and bacterial cells, the cellulose was washed with tap water and then boiled at 100°C for 20 min in 2 M potassium hydroxide. The cellulose was then washed to remove residual alkali and disrupted by means of a high speed homogenizer (Ultra-turrax, Janke and Kunkel, West Germany). The pooled suspension of cellulose was autoclaved and stored at room temperature and the dry weight of a sample determined gravimetrically.

Apparatus. Water-jacketted incubation vessels of 15 to 100 ml working volume, supplied by the Glassblowing Workshops of the CSIR, were used for all growth and enzyme experiments. The vessels were provided with ports for inlet and outlet of gas, a pH electrode and a Luer-Lok cannula for inoculation and withdrawal of samples. They were supported on a six-place, variable speed magnetic stirrer and water from a circulation thermostat was circulated through their jackets. The pH electrodes were linked to a six-channel pH meter and provision was made for bleeding NH_3-vapour from a lecture bottle into the gas supply of any vessel to restore the initial pH, if necessary. The assembled vessels were sterilized with ethylene oxide vapour for at least 16 h and then flushed with the appropriate gas phase.

Growth experiments. Fifty millilitre volumes of sterile, semi-defined medium based on that of Caldwell and Bryant (1966), but containing 0.1% (w/v) [^{14}C]-labelled cellulose as carbon and energy source, were dispensed by syringe into each vessel. The vessels were then equilibrated with a gas mixture of 95% CO_2 and 5% H_2 at 39°C before inoculation. At zero time each vessel was inoculated with 1 ml of a late log phase culture of R. flavefaciens FD1.

Enzyme incubations. A crude cellulase preparation from Trichoderma reesei C30 was donated by Dr T G Watson of the National Food Research Institute, CSIR, and Prof P J du Toit of the Orange Free State, kindly supplied us with small quantities of endo- and exocellulase fractions which had been isolated from T. reesei cellulase by gel filtration.

A range of enzyme preparations (0.25 mg to 1.25 mg dissolved in 200 µl each, of 0.05 M trisodium citrate buffer, pH 4.8) were added to 50 ml volumes of the same buffer, containing 0.1% (w/v) labelled cellulose in the case of the purified enzyme preparations, or 0.2% (w/v) for the crude enzyme preparations. The mixtures were incubated aerobically at 50°C.

Assay procedure. One millilitre samples were withdrawn from each reaction vessel immediately after enzyme addition or inoculation, and at regular intervals thereafter. Residual cellulose and, if present, bacterial cells were sedimented by centrifugation at 10 000 x g for 2 min in an Eppendorf microcentrifuge. Duplicate 200 µl aliquots of the supernatant were then added to 4 ml volumes of Scintillator 299 (United Technologies Packard) scintillation fluid and placed in a Searle Mark III Model 6880 scintillation counter for counting. After correction for background, the mean DPM value for each sample was plotted against incubation time.

RESULTS AND DISCUSSION

In order to demonstrate that this assay measures total cellulase activity and not just carboxymethylcellulase activity, solubilization of the [U-^{14}C]-labelled bacterial cellulose by purified exo- and endocellulase fractions isolated from T. reesei, separately and in combination, was monitored (figure 1). Solubilization of the cellulose by the endocellulase fraction alone showed very little activity as would be expected for a crystalline cellulose. The exocellulase fraction alone displayed greater solubilization activity. However, in the presence of both the endo- and exocellulases, the solubilization activity was greater than the sum of the individual activities. Thus demonstrating the synergistic effect between these two enzymes which is not observed when using an amorphous cellulose as substrate. Working with, inter alia, an unlabelled Acetobacter cellulose preparation, but measuring cellulase activity as the rate of production of reducing sugars, Henrissat et al. (1985) made very similar observations for highly purified exo- and endocellulases from T. reesei. We thus conclude that our assay method does measure total cellulase activity.

Further kinetic studies were conducted with a crude I. reesei cellulase preparation due to limited amounts of endo- and exocellulases at our disposal. A range of concentrations of this cellulase preparation from 5 to 20 µg/ml were incubated with 0.2% (w/v) [^{14}C]-cellulose and release of ^{14}C was monitored. The results are expressed as release of radioactivity (DPM/ml) versus incubation time (h) as shown in figure 2. All plots show a biphasic relationship with

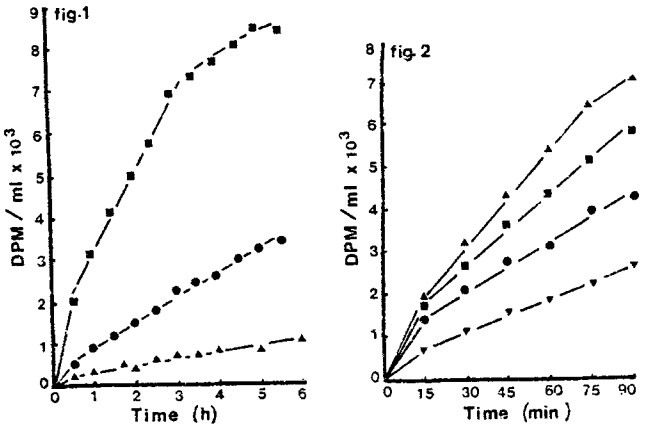

Fig 1. Release of ^{14}C from labelled bacterial cellulose following incubation with purified cellulase fractions isolated from I. reesei. Endocellulase, 98 µg/ml ▲; exocellulase 20 µg/ml ●; endocellulase, 98 µg/ml plus exocellulase, 20 µg/ml ■.

Fig 2. Solubilization of [U-^{14}C]-labelled bacterial cellulose by a range of concentrations of a crude cellulase preparation from I. reesei; 5 µg/ml ▼; 10 µg/ml ●; 15 µg/ml ■; 20 µg/ml ▲.

high initial rates of short duration. Thereafter a lower but constant rate was maintained provided that the substrate was present in saturating amounts. The high initial rate of solubilization is possibly due to the presence of a small fraction of cellulose of a lower degree of polymerization. Brown et al. (1976) found that under semi-anaerobic conditions A. xylinum produces a product which they suggest could be an intermediate cellulose polymer or a modified form of cellulose. These authors also report the presence of coiled lateral projections at the termini of cellulose ribbons which have been sheared from the bacterium during separation of the bacteria from the cellulose. These projections would most likely be more easily attacked by the enzymes than the cellulose ribbon itself. The initial rate of solubilization would therefore, not represent total cellulase

activity. But thereafter cellulose solubilization follows a constant rate which is dependent on the enzyme concentration.

Figure 3 shows linear and semi-logarithmic plots of the time course of release of radioactivity from labelled bacterial cellulose by a growing culture of R. flavefaciens. The linear plot shows the typical form of a batch growth curve. If it is assumed that the amount of

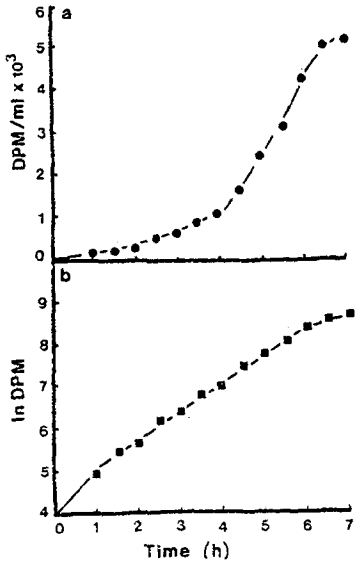

Fig. 3 Solubilization of [U-^{14}C]-labelled bacterial cellulose by a growing culture of R. flavefaciens. (a) a linear plot. (b) a semilogarithmic plot.

label liberated is directly proportional to the biomass produced, the slope of the linear region of the semi-logarithmic plot should give the specific growth rate of the culture in reciprocal hours. From the curve shown a growth rate of 0.69 h^{-1} was calculated. This is a high rate for growth on a crystalline cellulose. However, the high total pore volume and favourable pore size distribution of Acetobacter cellulose should facilitate access of the cellulases to the binding sites of their substrate (Grethlein, 1985) and thus allow higher rates of solubilization than substrates such as filter paper which are less penetrable to the enzymes.

We have described in the foregoing a simple assay suitable for studying total cellulase activity. The sensitivity of the assay can be varied by judicious choice of the specificity of the

D-[U-^{14}C]-glucose from which the cellulose is produced. There is freedom from interference by the presence of extraneous carbohydrates or strong reducing agents as may be found when using methods based on the measurement of reducing sugars. Finally, the method has wide applicability to growing microbial cultures, resting cells and crude or purified cellulase preparations of diverse origin.

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