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β-Glucanase Assay with Dyed Substrates

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Barley β-glucan stained with reactive dyestuffs was used as substrate for β-glucanase activity measurements. The method is suitable for both bacterial and malt enzymes.

Several reports have been published concerning enzyme activity measurements with substrates stained with reactive dyestuffs for textile industry. Dyed substrates have been applied for α-amylase [1–5] and protease [6,7] determinations. In most cases the substrates have been insoluble. The enzyme to be assayed produces soluble coloured molecules. Insoluble material is removed by centrifugation or filtration. The intensity of the soluble colour is a measure of the enzyme activity. It is also possible to use soluble dyed substrates, if the remaining high molecular compounds can be precipitated selectively. The soluble substrates are easier to handle and seem to increase the sensitivity of the assays.[5] In the present investigation barley β-glucan was stained with some reactive dyestuffs and used as substrate for β-glucanase determinations.

EXPERIMENTAL

Substrates. Barley β-glucan used in this investigation was prepared from barley flour as described by Schuster *et al.*[8] β-Glucan was stained with Reactonrot 2B (Geigy, Basel), using the method described by Babson [1] for α-amylase substrates. The substrate was purified by repeated ethanol precipitations, because gel chromatography did not give the desired purity.

10 g of β-glucan was dissolved in 200 ml of 1 N NaOH, and 2 g of Reactonrot 2 B was added to the solution. The mixture was stirred for 18 h at room temperature. The solution was then acidified with 3 N HCl, and the coloured β-glucan was precipitated by adding an equal volume of ethanol. The precipitate was collected by centrifugation and dissolved in hot water. The solution was cooled and precipitated again. This procedure was repeated until the supernatant was colourless. The final precipitate was washed with acetone, triturated and dried in a vacuum desiccator.

The β-glucan, dyed as described above, was mainly used in this investigation, but substrates stained with other textile dyestuffs appeared to be suitable, too. The staining with Cibachronblau F3G-A (Ciba, Basel) was carried out similarly as with Reactonrot 2B, but 0.1 N NaOH was used. Remazolbrillantblau R (Hoechst, Frankfurt am Main) was coupled to β-glucan, according to the method described by Stamm [9] for cellulose. This substrate was purified by washing with 50 % (v/v) ethanol.

β-Glucanase determination. Malt prepared from the Finnish six row *Pirkka* barley and a commercial bacterial enzyme preparation, Brew-n-zyme (N.V. Chemische Fabriek Naarden), were used as β-glucanase containing materials. The malt enzymes were extracted from 10 g of malt flour in 40 ml of 0.5 % NaCl solution at room temperature. The bacterial enzyme preparation was also dissolved in 0.5 % NaCl solution.

For β-glucanase determinations 3 ml of dyed β-glucan solution, 1 ml of Sørensen's phosphate buffer (*e.g.* pH 6.5), and 1 ml of the properly diluted enzyme solution were transferred into a test tube. The mixture was incubated at 30 °C and shaken every 10 min, because the substrate had a slight tendency to precipitate. The reaction was stopped by adding 5 ml of ice cold ethanol and placing the test tube in an ice bath. After 10 min

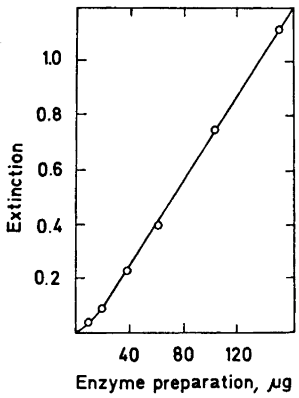

Fig. 1. Calibration curve for bacterial β-glucanase. pH 6.5, reaction time 40 min, substrate dyed with Reactonrot 2B.

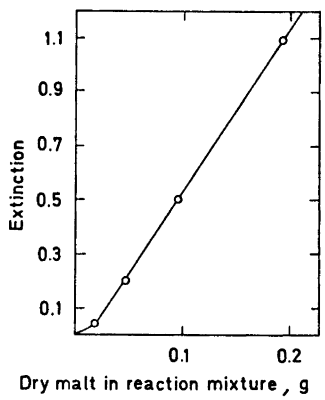

Fig. 2. Calibration curve for malt β-glucanase. pH 5.7, reaction time 40 min, substrate dyed with Reactonrot 2B.

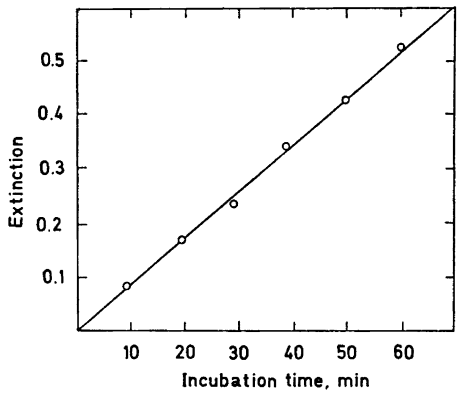

Fig. 3. The effect of the reaction time to the extinction values. Bacterial β-glucanase, pH 6.5, substrate dyed with Reactonrot 2B.

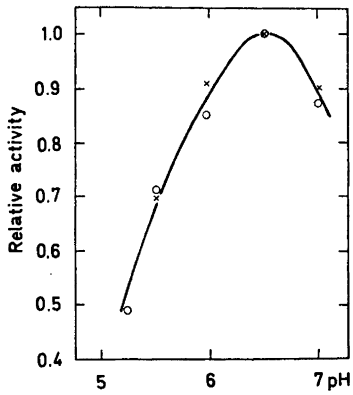

Fig. 4. pH-curve for the bacterial β-glucanase. The activity values were obtained with the dyed (Reactonrot 2B) substrate method (O) and with the method described by Moscatelli *et al.*[10] (×)

the contents were filtered through Whatman No. 1 filter paper, and the colour intensity of the filtrate was measured at 500 nm (the red substrate) or 595 nm (the blue substrate). Reagent blanks were prepared similarly but without the enzyme solution.

RESULTS AND DISCUSSION

All substrates gave linear calibration curves with the exception of very small enzyme activities (Figs. 1 and 2). The reason for this non-linearity at the initial phase of the reaction may be that the products of hydrolysis are not small enough to remain in the solution after addition of ethanol. On the other hand, a suitable dilution of the enzyme preparation should be used to keep the extinction value below 1.0. Above this value the results seemed to be less reliable.

The soluble colour produced is proportional to the reaction time (Fig. 3). Very small activities can thus be measured by using a prolonged incubation.

The coloured molecule covalently bound to β-glucan does not affect the pH-optimum of the β-glucanase. The pH-curve was determined using dyed and undyed β-glucan (Fig. 4). The method employed in the latter case was the one described by Moscatelli *et al.*[10]

The coloured substrate method is sensitive and convenient, and it is suitable for activity determinations from different materials such as malt and bacterial preparations. The extinction values obtained with various substrates are greatly affected by the degree of staining, *i.e.* the number of glucose or amino acid groups per one dyestuff molecule.[5,7]

For α-amylase or proteolytic enzymes, a suitable standard method can be used to achieve a conversion factor. The activities can thus be given in conventional units. However, there is no standardized method for β-glucanase. The method described by Moscatelli *et al.*[10] is based on the increase in the reducing power of the reaction mixture, and thus greatly affected by exo-enzymes. The methods based on the decrease in the viscosity of β-glucan solution are inconvenient and very sensitive to slight differences between the substrate batches.[11]

Studies on some details of the coloured substrate method, like standardization, effect of the degree of staining, and of the molecular size of the substrate, are in progress.

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