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Note

The role of water in the hydrolysis of cellulose under water-limiting conditions

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The growth of mildew on jute and consequent damage is a serious problem[1–4], causing[5] stains, loss in strength, a musty odour, and sometimes, bad hygiene. Humidity is necessary for the growth of mildew. Various antiseptics have been used to prevent growth of mildew[6], but such use may be objectionable, especially in food-packing bags. Moreover, the effect of some antiseptics may not be permanent[7]. Studies in our laboratory have indicated that drying of jute packs below 15% moisture is necessary to prevent fungal attack[8], suggesting that the action of cellulolytic enzymes is restricted by the non-availability of water. A study of the action of cellulase under water-limiting conditions should thus have close bearing on the mildew problem. This note examines the action of a pure cellulase [endo-(1→4)-β-D-glucanase] on its substrate under various levels of humidity.

RESULTS

Action of the dry enzyme on dry substrate after incubation at various relative humidities. — Dishes containing mixtures of dry cellulose (20 mg) and dry cellulase (2 mg) were incubated in chambers at different relative humidities (r.h.) for 8, 16, and 32 h. After the reactions, reducing sugars produced by hydrolysis of the cellulose were assayed and the reaction rates, expressed as absorbance at 520 nm per mg of protein, were plotted against time (Fig. 1). At 65% r.h., there was no detectable activity, even after 32 h. At 70% r.h. cellulase activity was observed only after 16 h; no measurable activity was seen after 8 h. At 75% r.h., hydrolysis began at the outset of the experiment and its rate was proportional to the time. This proportionality of rate with time was seen only up to 85% r.h. A diminished rate of hydrolysis observed at high r.h. values and at longer times of incubation may arise through *in situ* inhibition of the enzyme by the product. At 100% r.h., the rate became so high that it could not be compared on the same scale with rates observed at lower r.h. values.

Moisture-adsorption isotherms of cellulase and cellulose powder and their relation to the activity of the enzyme. — Dry cellulose powder (100 mg) and dry enzyme

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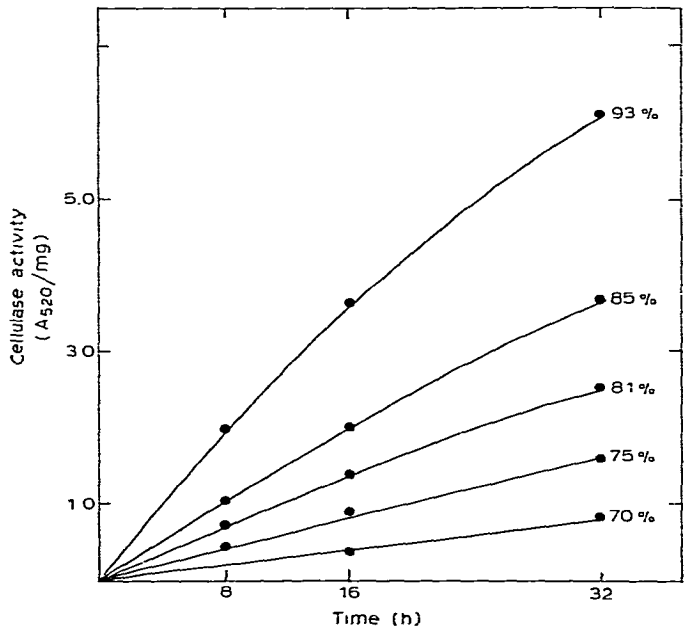

Fig. 1. Activity of dry cellulase at different relative humidities after incubation for various periods.

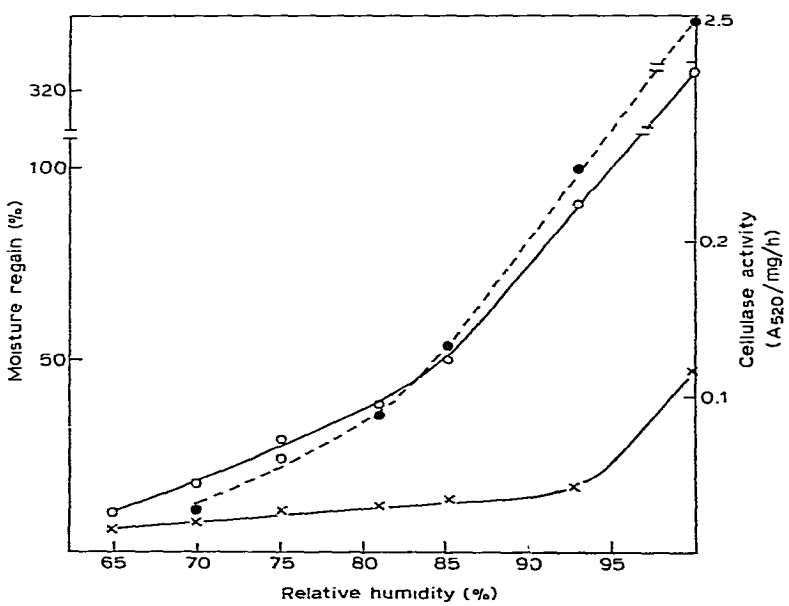

Fig. 2. Adsorption isotherms of the dry enzyme and substrate and their relation to the activity profile of the enzyme as at different relative humidities: ——○——○—— adsorption isotherm of cellulase powder; ——×——×—— adsorption isotherm of cellulose powder; ----●----●---- activity of enzyme as found at different relative humidities.

powder (5 mg) were incubated in separate bottles at different r.h. values at 40°. Their weights were recorded at regular intervals until constant weight was reached. The adsorption isotherms, as expressed by percent moisture regain, are plotted in Fig. 2. At 100% r.h., the enzyme protein was hygroscopic and its moisture regain exceeded 300%. Adsorption isotherms for both substrate and enzyme were linear between 65 and 93% r.h., with the slope for the substrate being lower than that of the enzyme. The profiles of enzyme activity, as observed at different r.h. values, are also plotted in Fig. 2. They were also linear, and their slopes were comparable with those for the adsorption isotherm of the enzyme protein, especially at low r.h. At higher r.h. values, the rates of hydrolysis increased very rapidly and deviated from the former slopes.

DISCUSSION

Figs. 1 and 2 show that the dry cellulase has no action below 70% r.h. The enzyme protein and Walseth[9] cellulose, kept at 75% r.h., regain 17 and 7.3% of moisture, respectively. These results support our previous finding[8] that jute must be stored below 15% moisture regain to avoid growth of mildew. The question arises as to what is the role of water under such limiting conditions. Firstly it may affect the enzyme molecule itself in that the active site of the protein may be conformationally stabilized by water. Secondly water serves as the reagent for the hydrolysis. It appears from Fig. 2 that the activity profile of the enzyme at different r.h. values follows more closely the adsorption isotherm of the enzyme powder than that of substrate. This result may indicate that limiting factor is the availability of water to restore the active site of the enzyme molecule, rather than its requirement for the stoichiometric reaction. The excess of moisture above this minimum may then be utilized for the hydrolytic reaction, and the rate increases as more water is supplied.

EXPERIMENTAL

Preparation and drying of substrate. — Phosphoric acid-swollen cellulose[9] was dried by keeping it under vaccuum over phosphorous pentaoxide for 4 weeks.

Preparation and drying of pure cellulase. — The preparation and subsequent purification of cellulase from *Aspergillus terreus* has been described earlier[10–12]. The purification was monitored by disc-gel electrophoresis[13] at pH 8.3. A 7.5% gel chromatogram of the purified protein revealed a single band plus a faint, minor band for hemicellulase. This purified protein (50 mg) suspended in 100 mL of water (containing 10 mL of 10mM acetate buffer, pH 5.0) was lyophilized and then dried under vacuum over phosphorus pentaoxide to constant weight.

Operation of the humidity chambers. — Chambers of different r.h. were set up by keeping aqueous salt solutions of different concentrations in desiccators at 40°. Dry enzymes, substrates, or mixtures thereof were kept in these chambers at 40° for the time-periods specified to assure uniform distribution of moisture.

Determination of rates of hydrolysis. — Dry cellulose (20 mg) and dry cellulase powder (2 mg) were mixed in a cyclo-mixer. The mixtures were then incubated at different r.h. values at 40° for various periods. At the end of each reaction, ice-cold water (5 mL) was added to each mixture and it was rapidly filtered. Aliquots (1 mL) of the filtrates were assayed by the Nelson–Somogyi reagent[14]. The rate of hydrolysis is expressed as colour developed (A_{520}) per mg of enzyme protein per h of incubation.

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