# OXIDATION: AN IMPORTANT ENZYME REACTION IN FUNGAL DEGRADATION OF CELLULOSE

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Received 7 November 1974

## 1. Introduction

The extracellular enzyme stystem utilized by *Sporotrichum pulverulentum* for the degradation of cellulose has been reported in a series of papers [1–5]. From the culture solution obtained by growing this fungus on powder cellulose as the sole carbon source, five *endo*-1,4-β-glucanases and one *exo*-1,4-β-glucanase have been purified and characterized and a strong synergistic action between these *endo*- and *exo*-glucanases demonstrated. Recently another enzyme, cellobiose: quinone oxidoreductase, was isolated from the culture solution of *S. pulverulentum* [6–8]. This enzyme reduces quinones in the presence of cellobiose which is in turn oxidized to cellobiono-δ-lactone. The enzyme is thought to be of importance in both cellulose and lignin degradation [9,10]. In this case the quinones are produced by phenol oxidases which oxidize phenols produced during lignin degradation. We have now discovered yet another enzyme in the culture solution of *S. pulverulentum* which is important for cellulose degradation. The enzyme oxidizes cellulose and its presence during cellulose degradation gives at least twice the extent of degradation given by the same mixture of *endo*- and *exo*-glucanases when the oxidizing enzyme is absent.

## 2. Results

Quantitative purification of the *endo*- and *exo*-glucanases from the culture solution of *S. pulverulentum* makes it possible to 'reconstruct' the culture solution using purified *endo*- and *exo*-enzymes, the reconstructed solution containing the same quantities of these enzymes as was present in the original culture solution. The ratio of the *endo*-1,4-β-glucanases to the *exo*-glucanase was known [3]. To test whether these enzymes were the only important enzymes, cellulose degradation by the artificial culture solution was compared with that caused by the original unfractionated concentrated culture solution. Both solutions contained the same amounts of *endo*- and *exo*-enzymes It should be mentioned that one of the *endo*-enzymes hydrolyses cellobiose and the amount of aryl-β-glucosidase activity in the culture solution is very low. As can be seen in table 1, the concentrated but unfractionated culture solution degraded 52.1% of the dewaxed cotton whereas the artificial mixture of the *endo*- and *exo*-glucanases degraded only 20.0%. This indicated that an additional enzyme important for the degradation of highly crystalline cellulose was present in the unfractionated culture solution. When this solution was incubated with nitrogen instead of air, the degree of cellulose degradation decreased from 52.1 to 21.5% (table 1). This result strongly suggested that the additional enzyme was of an oxidizing character. Neither *endo*- nor *exo*-glucanases alone can attack cotton to such an extent that a weight loss is discernible (table 1).

To confirm that oxygen was indeed consumed during cellulose degradation by the cell-free culture solution, unfractionated culture solution was concentrated 50 times by ultrafiltration (Amicon TC-ultrafiltration System, Model IB; PM-10 membrane) and 1.5 ml of the concentrated solution was pipetted into the chamber of an oxygen electrode (Rank Brothers, Cambridge, England). Addition of powdered cellulose (Munktells powder cellulose, No. 400, Grycksbo Pappersbruk, Stora Kopparbergs Bergslags AB, Grycksb


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Table 1
Degradation of cotton cellulose by enzymes from *S. pulverulentum*

| Tube number | Enzyme preparation | Cellulose degradation weight loss, % |
|---|---|---|
| 1 | Concentrated culture solution | 52.1 (oxygen atmosphere) |
| 2 | Concentrated culture solution | 21.5 (nitrogen atmosphere) |
| 3 | Mixture of *endo*- and *exo*-glucanases | 20.0 |
| 4 | *Endo*-1,4-β-glucanases | 0.0 |
| 5 | *Exo*-1,4-β-glucanase | 0.0 |

In each collodion tube (Membranfilter Gesellschaft, Göttingen, Germany) dewaxed cotton (10 mg) was suspended in 1.5 ml of the respective enzyme solutions (1–5) dialysed against sodium acetate buffer (pH 5.0, 0.05 M). A slow airstream was bubbled through a capillary into each tube, except for tube 2 which received a stream of nitrogen (first passed through a pyrogallol solution in alkali to remove $O_2$). The tubes then received the following enzyme solutions: 1. Unfractionated, concentrated, culture solutions [3] containing 50 units of *endo*-1,4-β-glucanase activity (for def. of these units cf. [12] and the corresponding amount of *exo*-1,4-β-glucanase, cf. [3,4]. 2. As under 1. 3. A solution containing a mixture of 50 units of purified *endo*-1,4-β-glucanases and the corresponding amount (cf. [3,4]) of purified *exo*-1,4-β-glucanase. 4. Fifty units of *endo*-1,4-glucanases. 5. An amount of *exo*-1,4-β-glucanase corresponding to 50 units of *endo*-1,4-β-glucanases. To avoid end product inhibition all tubes were dialysed against the same buffer containing 0.01% merthiolate, during reaction. The experiment was carried out at 28°C for 68 hr and the extent of degradation was determined gravimetrically.

Sweden) caused a rapid oxygen consumption. A consumption of oxygen was also observed when cellobiose, methyl-β-D-cellobioside, glucose, lactose or maltose were incubated with the culture solution under the same conditions.

Paper chromatographic analysis of the products from the enzymic degradation of cellulose revealed the presence of glucuronic and gluconic acids as well as neutral sugars. Glucose was also converted into glucuronic and gluconic acids during incubation with the culture solution. This indicates the presence of a glucose oxidase which produces gluconic acid and another oxidase which produces the uronic acid. The rate of cellulose degradation was not, however, altered by adding glucose oxidase (analytical grade, Boehringer Mannheim GmbH) to the artificial mixture of *endo*- and *exo*-glucanases previously described. The extent of degradation was 20% (table 1) irrespective of whether or not glucose oxidase was present.

The unfractionated culture solution contains *endo*- and *exo*-glucanases which produce cellobiose as well as glucose from cellulose. It was therefore necessary to free the culture solution of these enzymes to determine whether cellulose itself may be oxidized by the oxidizing enzyme. This purification was achieved by a four step procedure using columns of Sephadex A:50, polyacrylamide P-60, SP Sephadex and finally Concanavalin A Sepharose. The purified preparation, devoid of *endo*- and *exo*-glucanases, could oxidize cotton so that approximately 1% of the anhydro glucose units were converted into uronic acid moieties determined as described by Bylund and Donetzhuber [11].

To investigate the occurrence of the oxidizing enzyme in fungi considered to be strongly cellulolytic, three more organisms (*Polyporus adustus, Myrothecium verrucaria* and *Trichoderma viride*) were selected and grown under conditions similar to those for *S. pulverulentum* [3]. The results (table 2) demonstrate that cell-free concentrated culture solutions of all these fungi degrade twice as much cellulose in the presence of oxygen as in the absence of oxygen. The oxidative enzyme is relatively unstable and has not been found in any of the commercially available 'cellulase' preparations studied.

Table 2
Degradation of cotton cellulose by cell-free, concentrated culture solutions of four different cellulose degrading fungi in the presence and absence of oxygen

| Organism | Cellulose degradation weight loss, % | |
|---|---|---|
| | $O_2$-atmosphere | $N_2$-atmosphere |
| Sporotrichum pulverulentum[1] | 52.1 | 21.5 |
| Polyporus adustus[2] | 42.6 | 18.0 |
| Myrothecium verrucaria[2] | 33.6 | 17.0 |
| Trichoderma viride[3] | 20.0 | 10.0 |

[1] Culture solution concentrated 50 times. [2] Culture solution concentrated 30 times. [3] Culture solution concentrated 20 times. The fungi were cultivated as described for *S. pulverulentum* [3]. The cell-free culture solutions were concentrated by ultrafiltration and dialysed against sodium acetate buffer (pH 5.0, 0.05 M). Dewaxed cotton (10 mg) was added to the concentrated culture solutions (1.5 ml) and air or nitrogen continuously bubbled through the incubation mixture. For further experimental conditions, see text to table 1.

## 3. Discussion

The mechanism of fungal cellulose degradation has been much discussed, particularly the $C_1$-$C_X$ theory [13]. The original suggestion was that the cellulolytic organism possessed a $C_1$-enzyme (of unknown nature) which activated the cellulose so that the $C_X$-enzymes (*endo*-1,4-β-glucanases) could more easily hydrolyse the β-1,4-glucosidic bonds. It was later suggested that the $C_1$-enzyme was an *exo*-1,4-β-glucanase [14,15]. This is supported by Wood and McCrae [16] and Berghem and Pettersson [17]. It is now understood that the *endo*-1,4-β-glucanases act at random over the cellulose chain and the *exo*-1,4-β-enzyme acts on exposed chain ends splitting off cellobiose [16,17] or glucose and cellobiose [5], the *endo*-1,4-β-glucanases and the *exo*-1,4-β-glucanase having a strong synergistic action [5,16,17].

## 4. Conclusions

The discovery of cellobiose:quinone oxidoreductase [6,7] indicated that, at least when wood is the substrate, the mechanism of fungal cellulose degradation is not simply a synergistic action between *endo*- and *exo*-glucanases giving rise to glucose and cellobiose as final degradation products. Such a mechanism can only be valid when cellulose alone is present. The discovery of the oxidative enzyme reported here makes it clear that it is not only hydrolytic enzymes that are involved in cellulose degradation.

One may suggest that the role of the oxidizing enzyme is to oxidize cellulose by inserting uronic acid moieties and thus breaking hydrogen bonds between the cellulose chains. This causes swelling of the cellulose and makes the crystalline parts more accessible. This is fully in line with the old theory [13] that the cellulose is first activated so that its accessibility for hydrolytic enzymes is increased. The cellulose oxidizing enzyme is produced at low levels and probably does not produce a highly oxidized cellulose under natural conditions. Introduction of even a small number of uronic acid residues may, however, cause disorder in crystalline cellulose.

Work designed to evaluate in more detail the role of direct oxidation in the fungal degradation of cellulose and to achieve the purification and characterization of the *S. pulverulentum* cellulose oxidizing enzyme is now in progress. At present the enzymic reaction is not known with sufficient detail to justify the assignment of a systematic name to this enzyme.

## References

[1] Eriksson, K.-E. and Rzedowski, W. (1969) Arch. Biochem. Biophys. 129, 683–688.
[2] Eriksson, K.-E. and Rzedowski, W. (1969) Arch. Biochem. Biophys. 129, 689–695.
[3] Eriksson, K.-E. and Pettersson, B. Eur. J. Biochem. In press.
[4] Eriksson, K.-E. and Pettersson, B. Eur. J. Biochem. In press.
[5] Streamer, M. Eriksson, K.-E. and Pettersson, B. To be published.
[6] Westermark, U. and Eriksson, K.-E. (1974) Acta Chem. Scand. B. 28, 204–208.
[7] Westermark, U. and Eriksson, K.-E. (1974) Acta Chem. Scand. B. 28, 209–214.
[8] Westermark, U. and Eriksson, K.-E. Acta Chem. Scand. In press.
[9] Hiroi, T. and Eriksson, K.-E. Svensk Papperstidn. In press.
[10] Ander, P. and Eriksson, K.-E. Svenks Papperstidn. In press.

[11] Bylund, M. and Donetzhuber, A. (1968) Svensk Papperstidn. 71, 505–508.
[12] Almin, K. E., Eriksson, K.-E. and Jansson, C. (1967) Biochim. Biophys. Acta 139, 248–253.
[13] Reese, E. P., Siu, R. G. H. and Levinson, H. S. (1950) J. Bacteriol. 59, 485–497.
[14] Eriksson, K.-E. (1969) in: Cellulases and Their Applications (Gould, R. F., ed.) pp. 58–59, American Chemical Society Publications, Washington, D. C.
[15] Eriksson, K.-E. and Pettersson, B. (1972) in: Biodeterioration of materials, Vol. 2 pp. 116–120, Applied Sciences Publishers Ltd., London.
[16] Wood, T. M. and McCrae, S. I. (1972) Biochem. J. 128, 1183–92.
[17] Berghem, L. E. R. and Pettersson, L. G. (1973) Eur. J. Biochem. 37, 21–30.