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EFFECT OF CELLULASE SIZE REDUCTION ON ACTIVITY AND ACCESSIBILITY

Hsin Chih Chen and Hans E. Grethlein*
Dept. of Chemical Engineering,
Michigan State University
East Lansing, MI 48824, and
P.O. Box 27609, Lansing, MI 48909*
Michigan Biotechnology Institute

SUMMARY:

Reducing the size of crude cellulase from Trichoderma reesei with various proteases showed that while the β-glucosidase activity on CMC generally increased, the extent of hydrolysis of hardwood and pretreated hardwood decreased. The rank in the decrease in hydrolytic activity is the same as the rank in the decrease in enzyme adsorption on the hardwood or pretreated hardwood.

INTRODUCTION

A major problem encountered in carrying out enzymatic cellulose digestion is the low rate of hydrolysis. In the previous studies (Grethlein, 1985), it has been shown that the initial rate of hydrolysis of various species of wood is proportional to the available surface area in pores of the cell walls that are larger than enzyme size. While various pretreatments can effectively increase the rate of hydrolysis by increasing this available area (Grethlein, 1984), further rate increases are desirable for economic lignocellulose utilization. The other approach is to consider if it is possible to increase the rate of hydrolysis by increasing the accessibility with reduced enzyme size. This study evaluates the effect of decreasing the size of the cellulase by protease treatment on the hydrolytic activity and enzyme adsorption on substrates.

Although degradation of enzymes with proteolytic enzymes commonly leads to loss of activity, there are some cases where enzyme activity is maintained even though a considerable part of the enzyme is removed by digestion. In the early study of yeast enolase degraded by leucine aminopeptidase or carboxypeptidase A (Nylander and Malmstrom, 1959), the activity of this enzyme remains unchanged even with 80% of its amino acid removed. In another example in ribonuclease, the three amino acids at the c-terminal end (valine, serine, and alanine) can be removed by carboxypeptidase A or a single peptide bond can be split by subtilopeptidase A without any loss of activity (Dixan and Welb, 1964). Moreover, the original activity of papain may be maintained even though leucine aminopeptidase cuts almost 60% of papain size (Hill and Smith, 1960).

More recently, important results have been reported by Eriksson and Petterson, 1982. Firstly, the endo-1,4-β-glucanase activity could be increased sometimes up to 100-fold when blood serum was added to the culture solution of Phanerochaete chrysosporium. Secondly, when either or both protease I and II (both are purified from P. chrysosporium) are added to the cell-free culture solution, there is an increase in endo-glucanase activity by the factor of 10. Although Proteases I and II are not clearly identified, they claimed that protease I has similar functions as carboxypeptidase B while protease II is similar to chrymotrypsin. It is also mentioned that subtilisin could have an activating effect when added into an endo-1,4-β -glucanase solution from P. chrysosporium. By treatment of cellobiohydrolase I (CBHI) with papain for both heavy (30:1) and light (300:1) ratio, Tilbeurgh et al. (1986) recently reported that 15% of enzyme (CBHI) size has been cut. The study of activity shows that there is no activity loss against soluble substrates (such as chromophoric glycosidases) however, activity is completely lost within 1 hr for treatment with heavy enzyme ratio and 4 hours for light enzyme ratio against an insoluble substrate (such as avicel). In this paper, we focus on the effects of various proteases on the crude cellulase activity from Trichoderma reesei against insoluble mixed hardwood. As a point of comparison, we also evaluate the cellulase activity on soluble carboxymethglcellulose (CMC).

MATERIALS AND METHODS

Proteases and Enzymes:

Six kinds of proteases are choosen based on their commercial availability and previous review. Among them, chrymotrypsin, subtilisin and papain have been claimed to be effective in reducing enzyme size. Trypsin, pepsin and thermolysin are also choosen for this study. For each treatment, 5 mg various proteases are added in 0.44 g Novo celluclast CCN 3000 84/4 (cellulase) and treated in 1 ml pH 4.8 citrate buffer at 50°C for 24 hours. The enzyme solutions are used for measurement of SDS molecular weight, endo-1,4-β-glucanase activity, enzymatic hydrolysis on hardwood and enzyme adsorption on hardwood.

SDS Electrophresis and Molecular Weight Measurements:

Molecular weights of various solutions are determined by SDS-PAGE discontinuous electrophoresis (Laemmli procedure, 1970). It has been proved (Weber and Osborn, 1969) that molecular weights of most proteins could be determined by measuring the mobility in polyacryalamide gels containing SDS. Protein standards of known molecular weight are electrophoresed and their mobilities can be used to determine the mobility (Molecular weight) of an unknown protein. The results of cellulase treated with various proteases are shown in Figure 1.

Endo-1,4-β -Glucanase Activity Measurements:

Standard carboxymethyl cellulose (CMC) assay for cellulase is used (Mandel et al., 1976) to measure the endo-1,4-β -glucanase activity of the above solutions. Carboxymethyl cellulose is provided from Aqualon Company under the catalog number CMC 9M31F. The effects of various proteases on the endo-glucanase activity of cellulase are shown on Figure 2.

Insoluble cellulose hydrolysis measurements:

Mixed hardwood (90% birch and 10% maple) was obtained as a 60-mesh sieved wood flour from Wilner Wood Products, Norway, Maine. Enzymatic hydrolysis was performed on native and pretreated mixed hardwood. Pretreatment of mixed hardwood was performed in the batch reactor with 1% H_2SO_4 under the boiling condition for 5 hours. The pretreated wood is filtered, washed to neutral pH and used as a wet cake. Enzymatic hydrolysis was carried out using various protease treated solutions from previous section (0.44 g cellulase/flask) and 0.04 g β-glucosidase (Novozym, 188). The hydrolysis took place in the 250 ml Erlenmeyer flasks which were placed in a water bath shaker operated at 50°C and 200 rpm. Each flask contained two grams dry hardwood (native or pretreated) and 100 ml pH 4.8 citrate buffer. The reducing sugars were determined by DNS method.

Protein Adsorption Measurements:

Adsorption experiments were run at room temperature (24-28°C) and stirred at 200 rpm. Substrates (native and treated hardwood) are previously submerged in Deionized water and agitated on a shaker at 200 rpm and room temperature overnight. Various enzyme solutions from previous sections are added to various amounts of wet substrates (1.5 - 2.5 grams dry solids) in a pH 4.8 citrate buffer of a total volume of 100 ml. Clarified fluid samples were taken at 0, 10, 30, 60, 120 minutes. The amount of enzyme adsorbed was taken as the difference between the control (i.e., no wood present) and samples. Protein contents are measured by Lowry assay.

RESULTS AND DISCUSSION

All the proteases reduce the cellulase size (Figure 1). Among these, papain has the most significant reduction of cellulase size up almost to 50%. Thermolysis and subtilsin reduce the size in the range around 20% to 30%, while the other

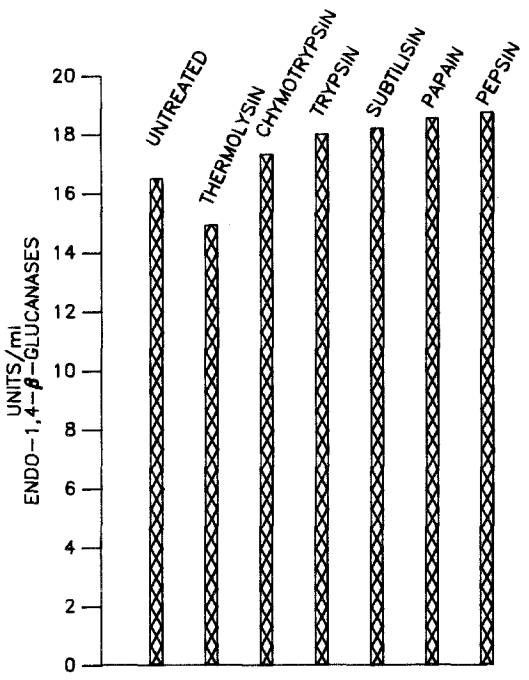


FIG 2 ACTIVITY OF ENDO-1,4-β-GLUCANASES FROM NOVO CELLULASE AFTER TREATMENT WITH VARIOUS PROTEASES

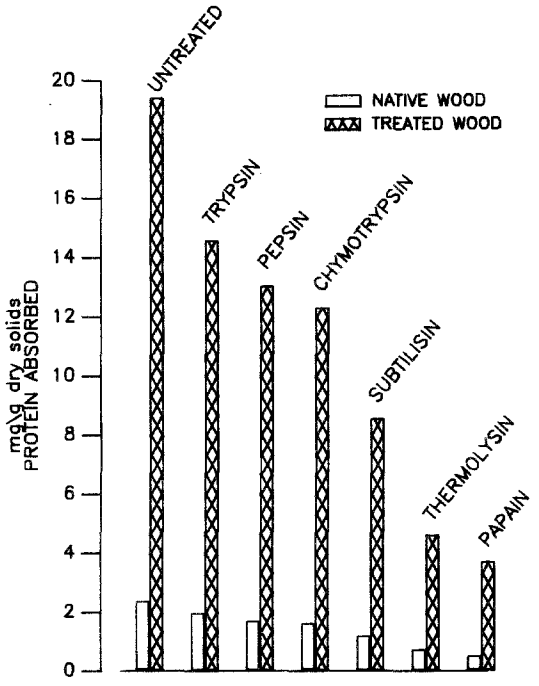


FIG 5 SATURATED PROTEIN LEVEL ABSORBED TO NATIVE AND PRETREATED MIXED HARDWOOD SAMPLES FOR THE ORIGINAL CRUDE CELLULASE AND FOR VARIOUS PROTEASE TREATED CELLULASES

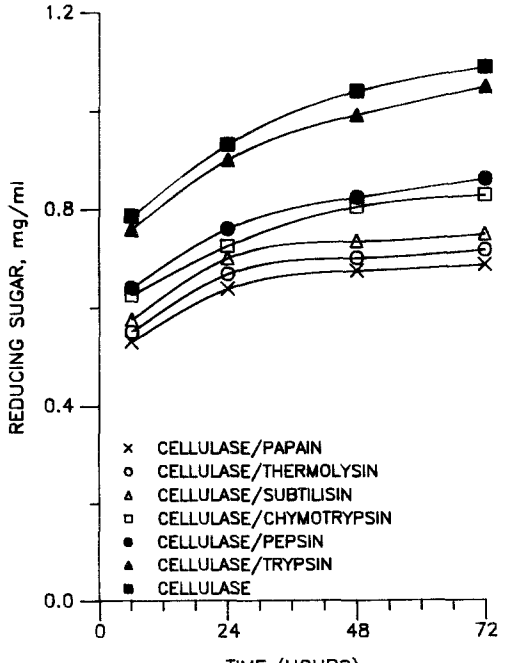


FIG.3 ENZYMATIC HYDROLYSIS OF NATIVE MIXED HARDWOOD BY CELLULASE AFTER TREATMENT WITH VARIOUS PROTEASES

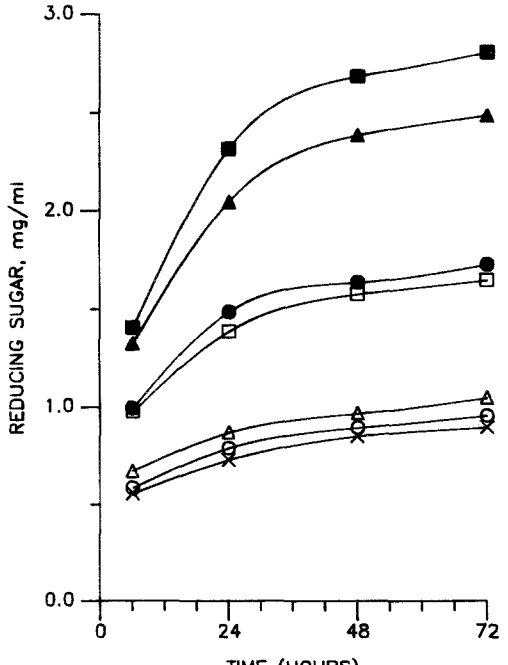


FIG.4 ENZYMATIC HYDROLYSIS OF TREATED HARDWOOD BY CELLULASE AFTER TREATMENT WITH VARIOUS PROTEASES

three proteases (pepsin, trypsin and chrymotrysin) have only a slight affect on the cellulase size. Although the protease treated cellulases is reduced in size, generally there is an increase in endo-1,4-β-glucanase activity. Treatment with pepsin increased endo-1,4-β-glucanase activity by a maximum of 14% (Figure 2). The other proteases have increased activity by factors of 5% to 12%. The only exception is thermolysin which has about a 10% decrease in endo-glucanase activity.

When these treated enzyme solutions were used on insoluble mixed hardwood, in the native or pretreated form, a surprising result has come out. All the treated enzymes decreased the rate and yield of reducing sugar production to some degree (Figures 3 and 4) compared to the original cellulase even though the endo-1,4- βglucanase activity is generally increased. Compared to the original cellulase, cellulase treated with trypsin has only 3% reduction of sugar production on native wood, but has almost 10% decrease on treated wood. Papain, on the other hand, reduces sugar production up to 40% and 70% on native and treated hardwood, respectively. However, for the corresponding protease treated cellulase, it is clear that pretreated wood produces larger sugar yield due to its larger available surface area compared to the native wood. The rank in decreasing order of hydrolytic activity on both native and treated wood is cellulase treated with trypsin, pepsin, chrymotrypsin, subtilisin, thermolysin and papain, respectively. This is the same rank as the size of the protease treated cellulase.

The reduction in enzyme size appears to remove the binding site of the cellulase which may account to its lower activity on wood. The adsorption of the various protease treated cellulases is demonstrated in Figure 5 for native and pretreatment wood. Note that the order in decreased cellulase adsorption is the same as the level of hydrolysis.

Contrary to our hypothesis, this study shows that radom size reductions in crude cellulase is not effective in increasing the rate of hydrolysis on insoluble cellulose. Since the active site and the binding site may be different, the cellulase of reduced size may still have equal or increased activity for a soluble substrate where accessibility is much less of a problem. Further investigations on the relationship between pretreatment of wood, and the mechanism of cellulase and ligninase attack are under investigation in our laboratory now.

REFERENCES

Dixan, M., and Welb, E.C. (1964). Enzymes 460.

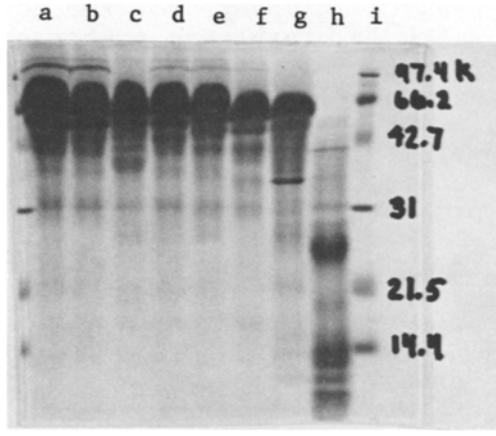
Eriksson, K.E., and Petterson, D.B. (1982). Eur. J. Biochem. 124, 635-642.

Grethlein, H.E. (1984). Adv. Biotech. 2, 43-62.

Grethlein, H.E. (1985). Bio/Technol. 3, 155-160.

Hill, R.L., and Smith, E.L. (1960). The Journal of Biological Chemistry. 235, No. 8, 2332.

Laemmli, U.K. (1970). Nature. 227,680.
Mandels, M, Andreotti, R. and Roche, C. (1976). Biotech. Bioeng. Sym 6. 21-34.
Nylanden, O., and Malmstrom, G. (1959). Biochimica ET Biophysica Acta. 34, 196.
Tilbeurgh, H.V., Tomme, P., Claeyssens, P., Bhikhabhai, R. and Pettersson, G. (1986). FEBS Letters. 204, No. 2, 223.



a: Cellulase
b: Cellulase in citrate buffer
c: Cellulase treated with pepsin in citrate buffer
d: Cellulase treated with trypsin in citrate buffer
e: Cellulase treated with chrymotrypsin in citrate buffer
f: Cellulase treated with subtilisin in citrate buffer
g: Cellulase treated with thermolysin in citrate buffer
h: Cellulase treated with papain in citrate buffer
i: Standard molecular weight sample

Figure 1. Effect of various proteases on the size reduction of cellulase.