The present study is an attempt to differentiate the roles of and B-glucosidase components in multiple forms of enzymes and their isoforms are generally distinguished and have clearly established that the extracellular cellulase can be used interchangeably for studies on the mechanism of action of a thiol-proteinase.

Comparison of the mode of action and site specificity of endo-(1,4)-β-D-glucanases of Penicillium pinophilum using normal, 1-3H-labelled, reduced and chromogenic cello-oligosaccharides

K. MAHALINGESHWARA BHAT*, ALISTER J. HAY*, THOMAS M. WOOD* and MARC CLAEYSSEN†

*Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB, U.K., and †Laboratorium voor Biochemische Wetenschappen, State University of Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium

Fractionation studies carried out in a number of laboratories have clearly established that the extracellular cellulase system of certain fungi contain endoglucanase, exoglucanase and β-glucosidase components in multiple forms [1]. These enzymes and their isoforms are generally distinguished and characterized using a variety of celluloses substrates, cello-oligosaccharides and their chromophoric derivatives [2, 3].

The present study is an attempt to differentiate the roles of the five major endoglucanases from a culture of the fungus Penicillium pinophilum using normal, 1-3H-labelled, reduced and chromophoric cello-oligosaccharides as substrates, and to examine the possibility that these substrates can be used interchangeably for studies on the mechanism of cellulase action.

Table 1. Number of methyl viologen-dependent nitrite reductase bands after native PAGE in gradient gels of samples dialysed or stored at 4°C

<table>
<thead>
<tr>
<th>Sample</th>
<th>Activity bands</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>1</td>
</tr>
<tr>
<td>Crude extract</td>
<td>2</td>
</tr>
<tr>
<td>Crude extract + 2.8 mm-iodeacetamide</td>
<td>1</td>
</tr>
<tr>
<td>Purified enzyme</td>
<td>1</td>
</tr>
</tbody>
</table>

*This sample was not dialysed or stored at 4°C.

Table 1 shows that the number of activity bands observed with the purified enzyme obtained by gel filtration coincided with that obtained by SDS/PAGE (54 kDa). Conversely, the purified enzyme contained a single activity band when native-PAGE was used, and it was only after a 4 days, a second minor activity band corresponding to a protein of 69 kDa appeared after the same type of electrophoresis. So, either the dialysis or the storage of the enzyme at 4°C could cause the partial dissociation of the dimer, thus obtaining two forms with methyl viologen-dependent activity, the dimer and the bigger subunit.

Despite the results suggesting the dimeric nature of the nitrite reductase from P. lirioideos, there are several findings opposing this idea. First, the molecular mass of the purified enzyme obtained by gel filtration coincided with that obtained by SDS/PAGE (54 kDa). Secondly, when the preparation which was dialysed or stored at 4°C was the purified enzyme, instead of the crude extract, only a single band of methyl viologen-dependent activity and protein appeared after the native/PAGE in gradient gel. Finally, the appearance of the second minor activity band was also prevented by the addition of 2.8 mm-iodeacetamide (a thiol-proteinase inhibitor) to the crude extract. This concentration of iodeacetamide did not affect the whole nitrite reductase activity. In conclusion, the nitrite reductase from P. lirioideos seems to be composed of a single polypeptide chain of 54 kDa.

This value is obtained by SDS/PAGE and gel filtration, although the enzyme shows an anomalous behaviour in native/PAGE. The appearance of two methyl viologen-dependent activity bands only occurs after native/PAGE of the crude extract in gradient gel and could be due to the action of a thiol-proteinase.

The molecular mass of this enzyme is similar to that reported for other cyanobacterial nitrite reductases [11–13], although a higher value of 68 kDa has been reported for the Anabaena cylindrica enzyme [10]. In agreement with most higher plant nitrite reductases [2–6], the cyanobacterial enzyme appears as a monomeric protein but with a slightly lower molecular mass.


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None of the five endoglucanases was active on cellobiose, but higher cello-oligosaccharides (Glc₂–Glc₃) were readily hydrolysed by all endoglucanases except endoglucanase I. Endoglucanases III and IV hydrolysed cellotriolose and higher cello-oligosaccharides, whereas endoglucanases I, V and II used, respectively, cellohexaose, cellopentaose and cello-tetraose as minimal length substrates [4].

H.p.l.c. analysis of the products of hydrolysis provided some interesting information. Notable was the release of cello-oligosaccharides as substrates the mode of action was demonstrated more precisely. Thus, endoglucanases III and IV apparently attacked all the glycosidic bonds of (1-3H)cello-oligosaccharides (Glc₂–Glc₃), but endoglucanases II and V

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were more specific. Bond-cleavage-frequency analysis revealed that the predominant product of hydrolysis of all substrates by endoglucanases I, II, and IV was \([^{1}H]\text{celllobiose; endoglucanase III, however, released mainly} \([^{1}H]\text{glucose from} \([^{1}H]\text{cellotriose and endo-}
\text{glucanase V produced equal amounts of} \([^{1}H]\text{celllobiose and}\n\text{[1-3]H}\text{cellotriose. Many of the endoglu-}
\text{nucrases of} \text{P. pinophilum} \text{appeared to be celllobio-}
\text{hydrolases with a preference for the reducing end of the}
\text{molecule. Attempts were made to deduce the mode of action of}
\text{the endoglucanases using reduced cello-oligosaccharides as sub-}
\text{strates. It was found, however, that the mode of attack on}
\text{these substrates was quite different from attack on normal}
\text{and radiolabelled cello-oligosaccharides. Thus, endoglu-}
\text{nucases III and IV, which were active on cellotriose,}
\text{showed no apparent action on cellotri-tol. More interest-}
\text{ingly, endoglucanase I, which was active on cellopentaose,}
\text{also hydrolysed cellopentaol, but endoglucanase V, which}
\text{hydrolysed cellopentaose, was unable to hydrolyse the corre-}
\text{sponding cellopentaol.}

Some clearly defined results were obtained using methyl-}
\text{umbelliferon (MeUmb)-cello-oligosaccharides as substrates.}
\text{Thus, none of the endoglucanase was active on MeUmb-}
\text{celllobioside but endoglucanases II, III, IV, and V were active on}
\text{MeUmb-cellotriose, and all five endoglucanases}
\text{hydrolysed cellopentoside and higher MeUmb-}
\text{cello-oligosaccharides. None of the endoglucanases released}
\text{free phenol from any of the MeUmb-cello-oligosaccharides}
\text{tested. A detailed study of the pattern of hydrolysis and}
\text{the determination of the kinetic constants revealed that the}
\text{internal glycosidic bonds of the MeUmb-cello-oligosaccharides}
\text{were the preferred sites of attack. In conclusion it would appear}
\text{that (a) cello-oligos-}
\text{accharide derivatives are good model substrates for charac-}
\text{terizing and distinguishing endoglucanases and for providing}
\text{limited information on the mode of action; (b) there is not}
\text{always a good correlation between attack on the cello-oligo-}
\text{saaccharide derivatives and the normal cello-oligo-}
\text{saaccharides; (c) the diversity of attack on the normal and}
\text{derivatized cello-oligosaccharides suggests that the various}
\text{endoglucanases may have specific roles to play in cellulose}
\text{hydrolysis.}

\text{We are grateful for the gift of radiolabelled cello-oligosaccharides}
\text{from Dr P. Biely, the technical assistance of Mrs S. Bhat, and}
\text{financial support by the European Communities (contract RNW}
\text{132 U.K.) and NATO (contract 066/85).}

\text{degradation of Wood Components} (Higuchi, K., ed.) pp.
\text{469–504, Academic Press, Inc., Orlando, Fl.}
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\text{Distribution of} \text{A}_{1}\text{-adenosine receptors, adenosine deaminase and} \text{5'-nucleotidase in brain and other tissues of the pig}

\text{VICTEN CASADO, TERESA MARTI,}
\text{JOSEPA MALLOL, M. CARMEN LLUIS,}
\text{ENRIC I. CANELA and RAFAEL FRANCO*}
\text{Departament de Bioquimica i Fisiologia, Unitat de}
\text{Bioquimica i Biologia Molecular A, Facultat de Quimica,}
\text{Universitat de Barcelona, Marti i Franques 1, Barcelona}
\text{08007, Catalonia, Spain}

\text{Introduction}

\text{Adenosine is now considered a regulator with multiple}
\text{actions in different tissues. For instance, in the nervous}
\text{system it depresses neuronal firing [1]. In other tissues the}
\text{actions exerted by the nucleoside are quite variable (see [2]).}
\text{These responses are mediated by specific receptors which}
\text{are of two classes:} A_{1}\text{ and} A_{2}\text{. One} \text{A}_{1}\text{mediates inhibition of adenylyl}
\text{cyclase (EC 4.6.1.1), whereas another} \text{A}_{2}\text{mediates stimulation of the enzyme. Interactions of adeno-}
\text{sin receptors with the adenylate cyclase system are possible}
\text{due to the existence of G-regulatory proteins (see [2]). In}
\text{other tissues the mechanisms of inactivation of adenosine after}
\text{its interaction with specific receptors: cellular uptake by means of a nucleo-}
\text{side transporter system and degradation by adenosine de-}
\text{aminase. In this report we have measured the} \text{[^{3}H]R-PIA-binding}
\text{capacity of different tissues and different brain areas}
\text{together with the activities of} \text{5'-nucleotidase and adenosine}
\text{deaminase. The aim has been to correlate the presence of}
\text{A}_{1}\text{-adenosine receptors with the enzyme which produces extracellular}
\text{adenosine and with the enzyme which cleaves it.}

\text{Methods}

\text{Adenosine deaminase and} \text{5'-nucleotidase activities as}
\text{well as} \text{[^{3}H]R-PIA-binding capacity were measured in mem-}
\text{branous preparations of all the tissues studied. Tissues were}
\text{homogenized in} \text{10 vol. of a solution of 0.25 m sucrose}
\text{in} \text{5 mm-Tris/HCl buffer, pH 7.4. Homogenates were}
\text{centrifuged at 105 000} \times \text{g for} \text{30 min at 4°C and the mem-}
\text{brane pellets were resuspended in} \text{10 vol. of 50 mm-Tris/HCl}
\text{buffer, pH 7.4, and centrifuged under the same conditions.}
\text{This washing was repeated once more before enzymic and}
\text{binding analysis. The maximum binding capacity of adenosine receptors was}
\text{determined by equilibrium binding isotherms of the

Abbreviation used: R-PIA, \text{R-N'-phenylisopropyladenosine.}
*To whom correspondence should be addressed.

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