THE CELLULASES OF *TRICHODERMA VIRIDE*

Mode of action and application in biomass conversion

Promotor : dr. W. Pilnik, hoogleraar in de levensmiddelenleer
Co-promotor: dr. ir. A.G.J. Voragen, wetenschappelijk hoofdmedewerker

G. BELDMAN

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DRUKKERIJ VAN DENDEREN B.V.

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Abstract

Beet pulp and potato fibre were liquefied and saccharified with a combination of cellulase from Trichoderma viride and pectinase from Aspergillus niger. Cell wall polysaccharides were hydrolysed extensively. The application of a packed column reactor, connected with a hollow fibre ultrafiltration unit was investigated for the enzymatic hydrolysis of beet pulp. A high degree of polysaccharide hydrolysis of spent grain by the action of cellulases was obtained after pretreatment with sulfuric acid or sodium hydroxyde. Essentially only monomeric sugars were present in the enzyme digest of the acid pretreated material. Six endoglucanases, three exoglucanases and a β-glucosidase were purified from a commercial cellulase preparation of T. viride origin. These enzymes were characterized with respect to their activities on several cellulosic substrates as well as chemical and physical properties. The β-glucosidase showed no cellobiase activity. Random and less random acting endoglucanases were distinguished. Adsorption, kinetic and synergistic behaviours of these enzymes were investigated on crystalline cellulose. It was shown that an optimal ratio of endoglucanase to exoglucanase was needed in order to obtain a maximal degree of synergism. This ratio could be related to the adsorption behaviours of the cellulases, suggesting that synergism between endoglucanases and exoglucanases takes place in very close cooperation at the surface of the cellulose particle. From activity experiments on xylan specific and non-specific glucanases could be demonstrated. However, the non-specific glucanases prefer to hydrolyse β-1,4-glucosidic linkages over β-1,4-xylosidic bonds.

Stellingen

1. Het door Ruy et al. geconstateerde synergisme tussen niet-geadsorbeerd endo-β-glucanase en door competitieve adsorptie vrijgekomen exo-β-glucanase geeft geen informatie over het synergisme in de geadsorbeerde fractie.

   Ruy, D.D.Y., Kim, C. and Mandels, M., Biotechnol. Bioeng. 1983, 26, 488-496

2. De constatering van Sprey en Lambert dat het met behulp van isoelectrisch focusing geisoleerde cellulase-xylanase-β-glucosidase bevattende complex van eiwitten homogeen blijkt te zijn bij herfocusing is het intrappen van een open deur.

   Sprey, B. and Lambert, C., FEMS Microbiol. Lett. 1983, 18, 217-222

3. De op basis van activiteit opgegeven opbrengsten van gezuiverde cellulases zijn niet relevant.

   Kittsteiner-Eberle, R., Hofelmann, M. and Schreier, P., Food Chem. 1985, 17, 131-142

4. De door Fujii en Shimizu gebruikte endo- en exo-β-glucanases zijn volstrekt onvoldoende gezuiverd voor het toetsen van het door deze onderzoekers gebruikte model dat enzymatische hydrolyse van oplosbare cellulose-derivaten beschrijft.

   Fujii, M. and Shimizu, M., Biotechnol. Bioeng. 1986, 28, 878-882

5. Men dient te beseffen dat tijdens de produktie van biet-suiker, bij onvoldoende controle, de pH en temperatuur in de diffusietoren vrijwel optimaal kunnen worden voor β-eliminatieve pectine-afbraak.

6. De in een Japans patent omschreven toepassing van cyclodextrine voor de bereiding van poedervormige whiskey moet worden gezien als een vorm van drankmisbruik.

   Jpn. Kokai Tokkyo Koko, JP 57.149.238

7. Er zullen vóór de ingebruikneming van bepaalde nieuwe biotechnologische processen internationale maatregelen moeten worden genomen om te voorkomen dat derde-wereld landen, zoals na de invoering van het isomerase-proces is gebeurd, weer het kind van de rekening zullen worden.

8. Het verdient aanbeveling het model van Wood dat een verklaring geeft voor synergisme tijdens enzymatische hydrolyse van onoplosbaar kristallijn cellulose te toetsen op de afbraak van natief zetmeel.

   Wood, T.M. and McCrae, S.T., Proc. Bioconvers. Symp. I.I.T., New Delhi, 1977, pp 111-141

9. Gezien de milieuvervuilende aktiviteiten en het gebruik van termen zoals bio-industrie lijkt de agrarische sector zich steeds verder te ontwikkelen tot een "volwaardige" industrietak met alle daarbij behorende negatieve aspecten.

10. Na de kernramp in Tsjernobyl is eens te meer duidelijk geworden hoezeer informatieverlening en democratie gekoppeld zijn.

11. Indien de vermindering van het begrotingstekort steeds meer wordt gezocht in bezuinigingen bij het onderwijs, kan het kabinet binnenkort ook het cellentekort opheffen door simpelweg tralies te plaatsen voor de ramen van de dan verlaten studentenflats.

Stellingen behorende bij het proefschrift "The cellulases of *Trichoderma viride*. Mode of action and application in biomass conversion" door Gerrit Beldman. Wageningen 25 november 1986.

aan mijn ouders,
aan Hannie

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Dankbetuiging

Allen, die op de een of andere wijze hebben bijgedragen tot de totstandkoming van dit proefschrift, wil ik hartelijk danken. Naast alle dienstverlenende afdelingen van het Biotechnion, ben ik aan enkele mensen in het bijzonder dank verschuldigd:

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Mijn moeder, die mij naar de H.B.S. stuurde en die - hoewel dat toendertijd niet als vanzelfsprekend gold - er geen enkel bezwaar tegen maakte dat ik ging studeren.

Maar zonder jou, Hannie, was het allemaal niet gegaan.

Contents

1 Introduction

Although only approximatively 0.1-0.3% of the solar energy, reaching the earth, is fixed through photosynthesis by green plants (1), the annual production of organic plant substance from CO_2-fixation is enormous and estimated to be about 10^{11} - 10^{12} tons (2, 3). Utilization of only a fraction of these materials could, at least partially, help to solve the problems of food and energy shortage.

About 40% of plant biomass consists of cellulose, the linear polymer of anhydro-D-glucose units, linked by β(1,4)-glucosidic bonds (Fig. 1). The degree of polymerization of native cellulose

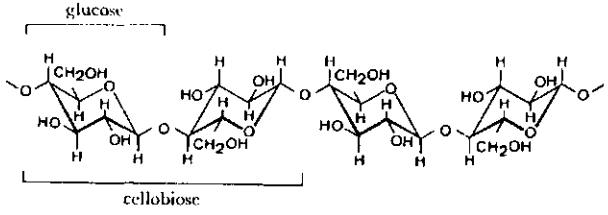

FIGURE 1. Conformation of the cellulose molecule.

is generally in the order of 10,000, which implies a molecular mass of 1.5×10^6 and a chain length of about 5 μm (4). The hydroxylgroups are in equatorial and the hydrogen atoms in the axial positions. Every other chain unit is rotated 180° around the main axis, which results in an unstrained linear configuration. Bundles of cellulose molecules, which are parallel ordered in native cellulose (5), form elementary fibrils with a partially crystalline structure caused by intra- and intermolecular hydrogen bonds. Aggregates of these fibrils (microfibrils) are the basis of the rigid structure of plant cell walls, where they are associated with a variety of other polymers, such as pectin, hemicellulose, lignin and structural proteins (6).

The plant cell wall consists of a middle lamella, primary wall and secondary wall. The amount of pectic substances, which are polygalacturonides with non-uronide carbohydrates covalently

bound to an unbranched chain of (1,4)α-D-galacturonic acid units, decreases in this order. The walls of soft, non-differentiated tissues are of the primary type, rich in hemicellulose and pectin and containing a type of cellulose which has a lower degree of polymerization and crystallinity than in the secondary wall (7).

The hemicelluloses are a group of polysaccharides, which contain pentoses (D-xylose, L-arabinose) and/or hexoses (D-galactose, D-mannose, D-glucose) as sugar residues. They do occur as homoglycans as well as heteroglycans, containing different types of sugar residues (8). In woody plant tissue the cellulose fibrils are closely associated with lignin, a highly polydisperse polyphenolic macromolecule of nine-carbon phenylpropane units.

The abundance of cellulose has been and still is a challenge for many biotechnologist to convert this material by the use of biochemical routes to glucose syrups. The literature on this field of research is extensively reviewed and deals about the quality and availibility of biomass (3, 9, 10), pretreatments and economics (1, 2, 9-12), process considerations (1, 9) and especially production and mode of action of cellulases (1, 3, 9, 12-21).

Cellulolytic enzymes are produced by a large number of micro-organisms, including fungi, actinomycetes, gliding bacteria (myxobacteria) and true bacteria (21). Bacterial cellulases are often cell-wall bound. Highly active cellulases are found in the culture media of fungi. Therefore these enzymes are studied extensively, especially with regard to their ability to degrade highly ordered crystalline cellulose. Thoroughly investigated are cellulases from Trichoderma spp. (T. viride, T. reesei and T. koningii), Fusarium solani, Penicillium funiculosum, Sporotrichum pulverulentum and Talaramyces emersonii (for references: see reviews 1-3, 9, 12-21).

The degradation of cellulose requires the participation of many enzymes. The first concept to explain the enzymatic degradation of crystalline cellulose was proposed by Reese et al. (22). A non-hydrolytic C_1 enzyme was thought to cause disaggregation of cellulose chains in crystalline cellulose, which results in a modified amorphous celullose. In turn, this cellulose can be attacked by hydrolytic C_x enzymes, producing soluble products.

$$\text{Crystalline cellulose} \xrightarrow{C_1} \text{amorphous cellulose} \xrightarrow{C_x} \text{soluble products}$$

In later studies it was found that a highly purified C_1 component is actually a hydrolytic enzyme, which releases a cellobiose molecule from the non-reducing chain-end (23, 24). This enzyme is classified as a cellobiohydrolase (1,4-β-D-glucan cellobiohydrolase EC 3.2.1.91), which acts co-operatively with two other types of enzymes in hydrolysis of native cellulose. These are: endo-1,4-β-glucanase (endo-1,4-β-D-glucan glucanohydrolase, EC 3.2.1.4) and β-glucosidase or cellobiase (β-D-glucoside glucohydrolase, EC 3.2.1.21). In some cellulase systems a glucohydrolase (1,4-β-D-glucan glucohydrolase, EC 3.2.1.74) is present. A model for the degradation of native cellulose by the action of these enzymes is proposed by Klyosov

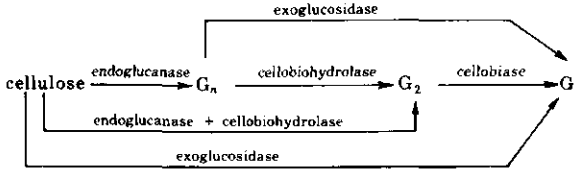

FIGURE 2. Model according to Klyosov for the breakdown of cellulose. G, glucose.

Cellobiose can be produced from cellulose by a sequential attack of endoglucanase and cellobiohydrolase via cello-oligosaccharides or by a combined action of these two enzymes which is thought to take place in very close co-operation at the surface of crystalline areas of the cellulose fibrils. This latter route is suggested to be responsible for the synergism, often observed in mixtures of endoglucanases and cellobiohydrolases (21). Cellobiase hydrolyses cellobiose, which is a strong inhibitor of both endoglucanase and cellobiohydrolase, to two glucose units. Direct formation of glucose can be established by exoglucosidase activity.

In the beginning of 1980 the Commission of the European Communities started to promote a research programme concerning the production and thermochemical and biological conversion of biomass. The main part of the research described in this thesis was carried out within the framework of this programme. The aim of the study was to apply polysaccharide degrading enzymes in the conversion of agricultural residues to fermentable sugar solutions. In this case bioconversion of by-products in the

beet-sugar and potato-starch industries (Chapter 2) as well as of beer brewers' spent grain (Chapter 3) were investigated.

Since cellulose is the main constituent of most plant biomass, special attention was paid to the mode of action of the cellulase complex present in a commercial preparation derived from Trichoderma viride. Although cellulases from this fungus have been studied extensively, there is still some debate on the mode of action of these enzymes on crystalline cellulose (9, 21). In order to investigate the role of the different cellulases present in the commercial preparation all endo-glucanase, exoglucanase and β-glucosidase activities were isolated (Chapter 4). Adsorption and kinetic behaviours of these purified enzymes as well as the synergism, observed during combined action of endoglucanase and exoglucanase (cellobio-hydrolase) on crystalline cellulose were studied (Chapter 5 and 6). Finally the non-specific mode of action of some the purified enzymes on arabino-xylan was investigated (Chapter 7).

REFERENCES

1. Tangu, S.K., Process Biochem. 1982, Mary/June, 36-45
2. Ryu, D.D.Y. and Mandels, M., Enzyme Microb. Technol. 1980, 2, 91-102
3. Bisaria, V.S. and Ghose, T.K., Enzyme Microb. Technol. 1981, 3, 90-104
4. Fan, L.T., Lee, Y.H. and Beardmore, D.H., Adv. Biochem. Eng. 1980, 14, 101-117
5. Sarko, A., Tappi 1978, 16, 59-61
6. Keegstra, K., Talmadge, K.W., Brauwer, W.D. and Albersheim, P., Plant Physiol. 1973, 51, 188-196
7. McNeil, M., Darvill, A.G., Fry, S.C. and Albersheim, P., Ann. Rev. Biochem. 1984, 53, 625-663
8. Aspinall, G.O., in The Polysaccharides, Pergamon Press, New York, 1970
9. Ladisch, M.R., Lin, K.W., Voloch, M. and Tsao, G.T., Enzyme Microb. Technol. 1983, 5, 82-102
10. Keenan, J.D., Process Biochem. 1979, May, 9-15
11. Ladisch, M.R., Process Biochem. 1979, Jan., 21-25
12. Mandels, M., ASM News 1981, 47, 174-178
13. Coughlan, M.P. and Folan, M.A., Int. J. Biochem. 1979, 10, 103-108
14. Ghose, T.K. and Ghosh, P., Process Biochem. 1979, Nov., 20-25
15. Mandels, M. and Andreotti, R.E., Process Biochem., 1978, May, 6-13
16. Gosoyr, J. and Eriksen, J. in Microbial Enzymes and Bioconversions (Rose, A.H. ed.), Academic Press, New York, 1980, p.p. 283-332

17. Shewale, J.G., Int. J. Biochem., 1982, 14, 435-443
18. Woodward, J. and Wiseman, A., Enzyme Microb. Technol. 1982, 4, 73-79
19. Montenecourt, B.S., Trends in Biotechnol., 1983, 1, 156-161
20. Lutzen, N.W., Nielsen, M.H., Oxenboell, K.M., Schulein, M. and Stentebjerg-Olesen, B., Phil. Trans. R. Soc. Lond., 1983, 300, 283-291
21. Wood, T.M., Biochem. Soc. Trans., 1985, 13, 407-410
22. Reese, E.T., Siu, T.G. and Levinson, H.S., J. Bacteriol. 1950, 59, 485-497
23. Halliwell, G. and Griffin, S., Biochem. J., 1973, 135, 587-594
24. Wood, T.M. and McCrae, S.I., Carbohydr. Res., 1977, 57, 117-133
25. Klyosov, A.A., Sinitsyn, A.P. and Rabinowitch, M.L. in Enzyme Engineering (Weetall, H.H. and Royer, G.P. ed.) vol. 5, Plenum Press, New York, 1980, p.p. 153-165

2 Application of cellulase and pectinase from fungal origin for the liquefaction and saccharification of biomass

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APPLICATION OF CELLULASE AND PECTINASE FROM FUNGAL ORIGIN FOR THE LIQUEFACTION AND SACCHARIFICATION OF BIOMASS

G. Beldman, F.M. Rombouts, A.G.J. Voragen and W. Pilnik

Department of Food Science, Agricultural University, Wageningen

Commercial cellulase [see 1,4-(1,3;1,4)-β-D-glucan 4-glucanohydrolase, EC 3.2.1.4] from Trichoderma viride and pectinase [poly (1,4-α-D-galacturonide) glycanohydrolase, EC 3.2.1.15] from Aspergillus niger have been applied to produce fermentation syrups from sugar-beet pulp and potato fibre. Cellulosic, hemicellulosic and pectic polysaccharides of these substrates were hydrolysed extensively. Recovery of enzymes has been investigated in a packed-column reactor, connected with a hollow-fibre ultrafiltration unit. Enzymes appeared to be stable in this type of reactor, although part of the enzyme activity was lost, especially by adsorption onto the substrate residue.

INTRODUCTION

Current agricultural sources of fermentable carbohydrates are sugar-beet, sugar-cane, corn, wheat, rice, potatoes and Jerusalem artichokes. In one way or another these raw materials must be processed to obtain fermentable sugars: the intracellular carbohydrate is extracted from the plant cells and, in the case of starch and inulin, the polysaccharides are hydrolysed to glucose and fructose, respectively.

The plant cells are surrounded by a structural tissue, the cell wall, which is built up of a thin primary wall and a thicker secondary wall with the middle lamella located between adjacent cells (1). Cellulose, hemicellulose, pectin and lignin are the main constituents of the plant cell wall.

Large quantities of plant cell-wall material become available as by-products in the beet-sugar and potato-starch industries. For instance, in the Netherlands 4.7×10^5 tons (dry matter) of beet pulp and 0.6×10^5 tons (dry matter) of potato fibre are produced.

The purpose of the present work is to investigate the enzymatic hydrolysis of these materials. Such a process is

attractive for two main reasons: first the production of fermentable sugars from the polysaccharides, and second the disruption of the cell-wall matrix, resulting in liquefaction of the materials and the release of intracellular carbohydrates.

A large spectrum of polysaccharide-degrading enzymes (cellulases, pectinases, hemicellulases) is needed for the bioconversion of plant material. Enzyme action is restricted by the accessibility of the substrate. The cellulases especially are hindered in their action on cellulose by the presence of the other polysaccharides (and lignin). Combinations of polysaccharide degrading enzymes are known to act synergistically in the degradation of the cell-wall matrix (2).

This paper deals with the application of commercial cellulolytic and pectolytic enzymes of fungal origin to hydrolysis of beet pulp and potato fibre. A hydrolysis process of beet pulp which can be operated on a continuous basis, with recovery of enzymes, was investigated in a column reactor connected to a hollow-fibre ultrafiltration unit.

MATERIALS AND METHODS

Biomass

Pressed and unpressed beet pulp (21.1 and 9.8% dry matter content, respectively) and potato fibre (15.9% dry matter content) were from Dutch factories.

Enzyme preparations

Commercial enzyme preparations, Maxazyme CL [cellulase, 1,4-(1,3;1,4)-β-D-glucan 4-glucanohydrolase, EC 3.2.1.4, from Trichoderma viride], Rapidase C80 [pectinase, poly(1,4-α -D-galacturonide) glycanohydrolase, EC 3.2.1.15, from Aspergillus niger] and Hazyme Rapidase [α-amylase, 1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1, from Aspergillus niger] were kindly provided by Gist-Brocades, Delft, the Netherlands.

Analysis methods

Reducing sugars were measured according to Somogyi (3). The galacturonide content was estimated using the method of Ahmed and Labavitch (4). The neutral sugar composition was determined by gas chromatography after Saeman hydrolysis (5) and derivatization to alditol acetates (6). Soluble neutral sugars were analysed by HPLC using a Lichrosorb 10 NH_2 column. Cellulose determination was done according to the method of Updegraff

(7). The Kjeldahl method was used for protein analyses. Starch content was measured using the Boehringer enzyme kit (Boehringer-Mannheim, FRG).

Liquefaction

Enzymic liquefaction of beet pulp (unpressed) and potato fibre was followd by measurement of consistency changes in a thermostatically controlled rotating reaction vessel of a Brabender Viskograph (Brabender, OHB, Duisburg, FRG), at 40°C and 100 rev min^{-1}, for 4 h. The pulp was ground in a meat-grinder. To fall within the resistance range capable of being measured by the instrument, 70 g water had to be added to 100 g pulp.

The pulp was incubated with a combination of cellulase and pectinase at pH 5.2 and 3.8. The contribution of individual cellulase and pectinase preparations to changes in consistency was investigated at pH 3.8. The cellulase concentration was 1.08 mg ml^{-1} and the pectinase concentration 1.35 mg ml^{-1}.

Saccharification

Beet pulp (unpressed) and potato fibre were saccharified in a stirred batch reactor at 40°C and pH 4.0 for 24 h. The beet pulp was ground and cellulase and pectinase were added to a concentration of 2.0 and 2.5 mg ml^{-1}, respectively.

Saccharification of potato fibre was carried out after a gelatinization pretreatment of the starch (15 min at 85 °C). Enzyme concentrations were (mg ml^{-1}): celluase, 2.0; pectinase, 2.5; and α-amylase, 0.5. To prevent microbial growth NaN_3 was added to a concentration of 0.01%.

Packed-column reactor experiments

Saccharification of pressed beet pulp and potato fibre was studied in a packed-column reactor, connected with a hollow-fibre ultrafiltration unit (Amicon CH4, H1P5 cartridge, 5000 MW cut-off). This system (Figure 1) is capable of continuous operation, with recovery and re-use of the enzyme. The column had a volume of 2.56 litre and contained 1850 g pulp which was brought to pH 4.0 with 10% HCl. An enzyme solution, which gave a cellulase concentration in the system of 5.68 mg ml^{-1} and a pectinase concentration of 7.14 mg ml^{-1}, was pumped up into the column and recirculated. Percolation pump 1 was operated at 1.01 litre h^{-1}. Column and enzyme vessel were kept at 40°C. The ultrafiltration unit was exposed to room temperature.

Enzyme inactivation was studied in the column/hollow-fibre reactor in the presence and absence of beet pulp. Samples were assayed for their enzyme activities after removal of the sugars with Amicon membrane cones, type CF 25.

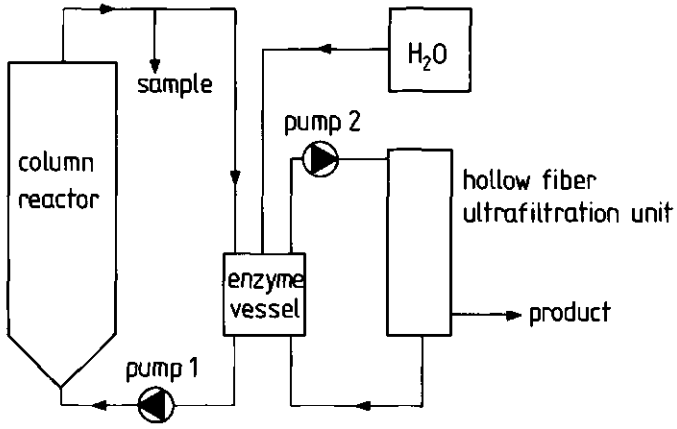

Fig. 1. Experimental set-up for saccharification of beet pulp in a column/hollow-fibre membrane reactor.

Enzyme acitivity assays

Exo-1,4-β-D-glucanase activity in the commercial enzyme preparations was measured by the increase in reducing sugars from Whatman no. 1 filter paper (filter paper activity, FPA). Pieces of 2 x 2 cm (33.2 mg) were incubated with different amounts of enzyme in 6 ml 0.1 M sodium acetate buffer, pH 4.0 at 40°C. Activity of the amount of enzyme which gave 10% hydrolysis after 2 h incubation was determined using the Nelson-Somogyi method.

Endo-1,4-β-glucanase activity was measured viscometrically with carboxymethyl cellulose (type Akucell AF 0305, Akzo, Arnhem, the Netherlands; carboxymethyl cellulase, CMCase activity) at 30°C according to Almin and Eriksson (8), using a 0.25% substrate concentration.

Activity on pectin was determined by the same viscometric method, using 0.25% pectin with a degree of esterification of 38% (Obipectin Ltd, Bischoszell, Switzerland; pectinase activity), at pH 4.0 and 30°C.

Residual cellulase activity in the packed-column reactor was measured on Avicel (Type SF, Serva, Heidelberg, FRG; avicelase activity), for 20h, at 30°C in 0.05 M sodium acetate buffer, pH 5.0, using a 0.5% substrate suspension. After centrifugation the supernatant was analysed for reducing sugars.

Polygalacturonase, pectin lyase, pectinesterase and β-D-glucosidase were measured according to Voragen et al. (2) All activities were expressed in International Units (U) per gram of enzyme preparation, with the exception of CMCase and pectinase activities, which are expressed as rate of change in specific fluidity, $\Delta\Phi_{sp}$ min^{-1}.

RESULTS

Beet pulp and potato fibre were analysed for their carbohydrate compositions to give an indication of the kinds of enzymes to use, as well as to give a basis for assessing changes in sugar composition after enzymatic hydrolysis. The commercial cellulase and pectinase preparations were screened for their different enzyme activities. Optimum conditions with respect to pH and enzyme composition for the liquefaction of beet pulp and potato fibre were measured viscometrically. Pectinase and cellulase were used separately, as well as in combination.

Polysaccharide hydrolysis limits and sugar yields were determined in a stirred batch reactor and compared to the results obtained in a column hollow-fibre reactor, which was applied to recover enzymes.

Analysis of biomass

Table 1 gives the chemical composition of the biomass used in the present experiments. The main polysaccharides in beet pulp are cellulose, pectin and a hemicellulose fraction consisting predominantly of arabinose. There is some residual sucrose left in the pulp, especially in the unpressed sample.

Potato fibre has a similar composition. L-Arabinose and D-galactose are the main hemicellulosic sugars. The fibre contains a considerable amount of starch.

The amount of lignin was not determined, but is known to be low (2-4%) in similar materials (9).

Table 1. Chemical composition of beet pulp and potato fibre.

	Beet pulp (% of dry matter)		Potato fibre (% of dry matter)
	Unpressed	Pressed	
Cellulose	18.7	18.7	26.0
Pectin	11.7	15.0	15.3
Hemicellulose			
Araban	14.4	13.2	3.9
Xylan	0.8	1.2	1.8
Mannan	0.0	0.4	0.3
Galactan	2.9	2.8	7.9
Sucrose	17.3	5.8	0.0
Starch	0.0	0.0	17.1
Protein	5.1	6.0	14.9
Ash	9.0	11.8	5.2

Table 2. Protein and enzyme levels in commercial cellulase from Trichoderma viride and pectinase from Aspergillus niger.

	Enzyme preparation ($U\ g^{-1}$)	
	Cellulase	Pectinase
FPase	39.3	0.0
CMCase[a]	750.0	344.0
β-D-Glucosidase	32.4	120.0
Pectin lyase	0.0	50.4
Pectinesterase	0.0	767.0
Polygalacturonase	0.0	421.0
Protein[b]	337.0	180.0

[a] Arbitrary units (min^{-1})
[b] mg g^{-1} enzyme preparation

Activities of commercial enzyme preparations

Exo-1,4-β-D-glucanase (FPase) activity (FPA) was only found in the cellulase preparation (Table 2). No pectinase concentration was high enough to obtain 10% hydrolysis of filter paper within 2 h. Endo-1,4-β-D-glucanase (CMCase) and β-D-glucosidase activities were found in both preparations, with the pectinase preparation having a much higher β-D-glucosidase level. No pectolytic activity was found in the cellulase preparation.

Liquefaction

Treatment of beet pulp with a combination of cellulase and pectinase at pH 5.2 showed an intermittent delay in the viscosity decrease (Figure 2). After 2 h the process of liquefaction accelerated, however, and a simultaneous pH shift from pH 5.2 to 3.8 was observed (data not shown). The decrease in pH can be explained by assuming a de-esterification of pectin by pectinesterase action. The pH effect was confirmed by the fast initial decrease in viscosity of pulp, which was brought to pH 3.8 prior to enzyme addition.

Incubation of pulp with cellulase and pectinase separately showed that the pectinase preparation causes a dramatic viscosity loss (Figure 2) and that optimum action occurs in an acidic pH (Figure 3).

Essentially the same results were obtained from liquefaction experiments with potato fibre: the combination of cellulase and pectinase induced the fastest decrease in viscosity, but the pectinase preparation was largely responsible for the effect (results not shown).

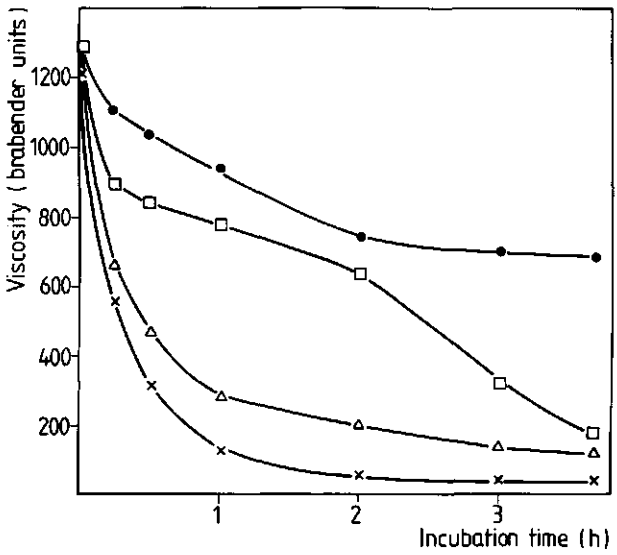

Fig. 2. Decrease of viscosity of sugar-beet pulp, treated with pectinase (1.35 mg ml^{-1}), cellulase (1.08 mg ml^{-1}) and a combination of these enzymes, at different pH values. □, Cellulase + pectinase, pH 5.2; ●, cellulase, pH 3.8; △, pectinase pH 3.8; ×, cellulase + pectinase, pH 3.8.

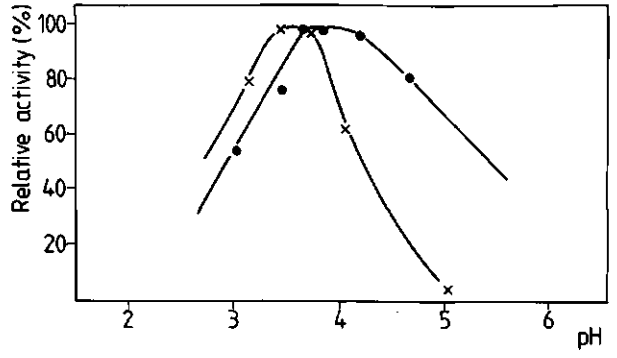

Fig. 3. Optimum pH for liquefaction of beet pulp, measured with viscograph and optimum pH for polygalacturonase. ×, Activity on beet pulp; ●, activity on polygalacturonic acid.

For these reasons all other liquefaction and saccharification experiments were carried out at pH 4.0.

Saccharification

Figure 4 shows the increase in reducing sugars from unpressed

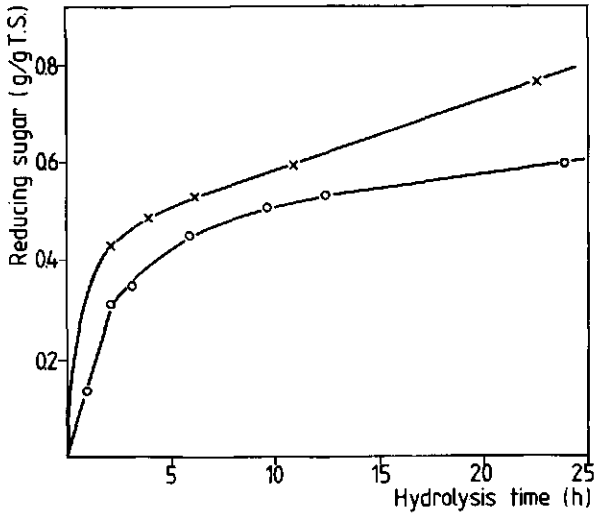

Fig. 4. Reducing sugar released from beet pulp and potato fibre during saccharification with cellulase (2.0 mg ml^{-1}) and pectinase (2.5 mg ml^{-1}). ×, Beet pulp; o, potato fibre.

beet pulp and potato fibre as a function of hydrolysis time. Almost 80% of the total solids of beet pulp were brought into solution as reducing sugars. Potato fibre gave 60% conversion of total solids to reducing sugars.

Polysaccharide hydrolysis limits were calculated from the carbohydrate analysis of the residues. More than 90% of beet pulp polysaccharides were hydrolysed (Table 3). Polysaccharide hydrolysis limits of potato fibre were lower for cellulose and pectin. However, starch and the hemicellulose polymers of L-arabinose and D-galactose were also extensively hydrolysed.

Table 3. Enzyme hydrolysis limits of main polysaccharides of beet pulp and potato fibre.

	Substrate (% hydrolysis)	
Polysaccharide	Beet pulp	Potato fibre
Cellulose	91.0	75.0
Pectin	91.0	58.0
Hemicellulose		
Araban	93.0	93.0
Galactan	90.0	96.0
Starch	0.0	99.0

Table 4. Sugar composition of enzyme digest after incubation of beet pulp and potato fibre with cellulase and pectinase.

Sugar	Substrate (g/g dry weight of substrate)	
	Beet pulp	Potato fibre
D-Glucose	0.38	0.45
D-Fructose	0.16	0.00
L-Arabinose	0.17	0.04
D-Galactose	0.02	0.06
Uronic acid	0.15	0.11

HPLC-analysis showed that almost all released sugars were in monomeric form; no cellobiose could be detected. Sucrose from beet pulp was inverted to D-glucose and D-fructose. The final product had a sugar composition as given in Table 4. D-Glucose, D-fructose, L-arabinose and D-galactose are the main sugar monomers from neutral polysaccharides. Uronic acid is the hydrolysis product from pectin molecules.

Packed-column reactor experiments.

The cumulative sugar production from pressed beet pulp during

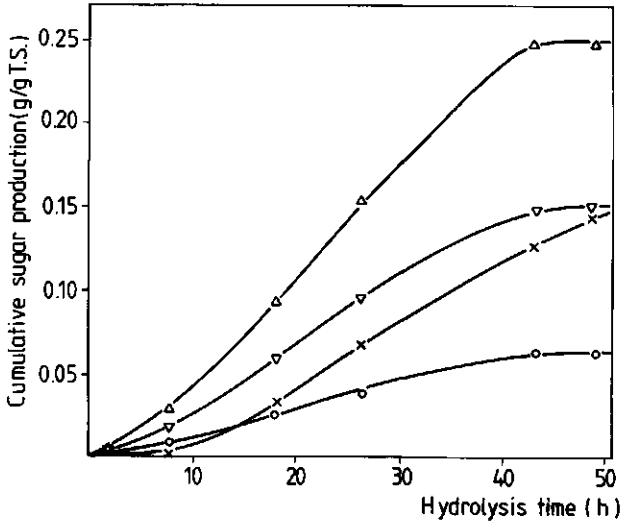

Fig. 5. Cumulative sugar production from pressed beet pulp during enzymatic saccharification in the column/hollow-fibre membrane reactor, Δ, D-Glucose; ∇, uronic acid; ×, L-arabinose; ○, D-fructose.

enzymic treatment in a column/hollow-fibre membrane reactor is shown in Figure 5. Hydrolysis was followed for 48 h. Sugars were measured at the ultrafiltration outlet. Hydrolysis limits for cellulose and pectin in the column reactor were calculated from the polysaccharide content of the residue and found to be similar to those in the batch reactor: cellulose was 89% hydrolysed and pectin 95%.

Enzyme inactivation in this type of reactor was studied in the presence and absence of beet pulp (Table 5). Residual enzyme acitvity measured in the absence of beet pulp gives an indication of the amount of enzyme which is inactivated by temperature and shear. In the presence of beet pulp the additional amount of enzyme, lost by adsorpion to the substrate, is also measured. Cellulase activity (avicelase, CMCase) was stable to shear and heat. Somewhat more pectinase activity was lost under these circumstances. The additional losses of avicelase and pectinase activities in the presence of substrate were 31 and 24%, respectively while β-D-glucosidase activity seemed to be stabilized in the presence of substrate.

Treatment of potato fibre in the column reactor gave poor results. The soft structure of the fibre caused bad flow characteristics of the column when the enzyme solution was pumped through.

Table 5. Residual enzyme activity in column/hollow-fibre reactor after 48 h of operation with or without beet pulp.

	Residual activity (% of original activity)	
Enzyme	Absence of substrate	Presence of substrate
Avicelase	82.0	50.8
CMCase	80.0	72.0
β-D-Glucosidase	84.6	98.0
Pectinase	66.7	42.9

DISCUSSION

The results presented in this paper indicate that commercial polysaccharide-degrading enzymes can be used to convert some solid plant biomass to fermentable sugar solutions. A combination of pectolytic and cellulolytic enzymes was able to produce monomeric sugar solutions from beet pulp and potato fibre. Cell-wall polysaccharides from beet pulp were extensively hydrolysed: more than 90% conversion was obtained. Cellulose and

especially pectin from potato fibre were somewhat more resistant to enzymatic hydrolysis. The basis for this resistance of potato fibre is not clear. Lignin may play a slight role, though its influence must be limited because it is a minor component. Cellobiose is known to be a strong inhibitor of cellulase. Since Trichoderma cellulase preparations are mostly deficient in cellobiase activity, addition of Aspergillus cellobiase reduces cellobiose levels and increases glucose content in the digest (10). This is confirmed by the experiments described in this paper: by the use of a combination of Trichoderma cellulase and Aspergillus pectinase with a high β-D-glucosidase level, no significant amounts of cellobiose accumulated during hydrolysis of beet pulp and potato fibre. Therefore, the lower extent of cellulose hydrolysis in potato fibre was apparently not caused by product inhibition. Probably the cellulose fibrils were still shielded by the pectin molecules which were not degraded by the pectic enzymes. The low degree of hydrolysis of this pectin may be caused by its high degree of acetylation (unpublished results).

There is a synergistic action of cellulases and pectinases in plant biomass conversion. Consistency measurements of beet pulp and potato fibre showed that the first step of the process, the disintegration of the plant cell walls, results in liquefaction of the material. This process is optimal at pH 3.5-4.0 and is mainly caused by the polygalacturonase from the pectolytic enzyme which has its optimum in the same region, while pectin lyase has its optimum at pH 6 (11). In a second step, the extensive hydrolysis of the cell wall polysaccharides, a cellulase preparation with high activity on crystalline cellulose (FPA) was needed.

The use of beet pulp and potato fibre, substrates with low lignin contents, gave high yields in cellulose hydrolysis (75-90%). It is known that in material with a high lignin content much lower cellulose conversions are obtained. Expensive treatments such as ball milling (12), attritor milling (13), compression milling (14), acid and alkaline pretreatment (15), steam explosion (16) or treatment with organic solvents (17) have to be used to raise the yield.

Results of the hydrolysis of beet pulp in a packed-column/hollow-fibre reactor showed the possibilities for a continuous process. The enzymes are kept in the reactor, the produced sugars leave the reactor through the ultrafiltration membrane and substrate is fed continuously e.g. by a cascade of reactors.

Simple operation of this type of reactor is posible because no stirring device is needed and a high solid/liquid ratio can be obtained. Recovery of enzymes by ultrafiltration appeared to be attractive because the enzymes were rather resistant to inactivation under these circumstances. However, part of the enzyme activity, especially avicelase activity, was lost by adsorption on the residue. Our results (Chapter 4) and those of others indicate that adsorbed cellulase can be recovered by a pH shift (18) to the alkaline region (pH 10) or by the use of surfactant (19).

REFERENCES

1. Keegstra, K., Talmadge, K.W., Bauer, W.D. and Albersheim, P., Plant Physiol. 1973, 51, 188-196
2. Voragen, A.G.J., Heutink, R. and Pilnik, W., J. Apll. Biochem. 1980, 2, 452-468
3. Somogyi, M., J. Biol. Chem. 1952, 195, 19-23
4. Ahmed, A.E.R. and Labavitch, J.M., J. Food Biochem. 1977, 1, 361-365
5. Saeman, J.F., Moore, W.E. and Millet, M.A., Methods Carbohydr. Chem. 1963, 3, 54-69
6. Jones, T.M. and Albersheim, P., Plant Physiol. 1972, 49, 926-936
7. Updegraff, D.M., Anal. Biochem. 1969, 32, 420-424
8. Almin, K.E. and Eriksson, K.E. Biochim. Biophys. Acta 1967, 139, 238-247
9. Voragen, F.G.J., Timmers, J.P.J., Linssen, J.P.H., Schols, H.A. and Pilnik, W., Z. Lebensm. Unters. Forsch. 1983, 177, 251-256
10. Ruy, D.D.Y. and Mandels, M., Enzyme Microb. Technol. 1980, 2, 91-102
11. Rombouts, F.M. and Pilnik, W. in Microbial Enzymes and Bioconversions (Rose, A.H. ed.), Academic Press, New York, 1980, pp. 227-281
12. Mandels, M., Hontz, L. and Nystrom, J., Biotechnol. Bioeng. 1974, 16, 1471-1493
13. Ryu, S.K. and Lee, J.M., Biotechnol. Bioeng, 1983, 25, 53-65
14. Ryu, D.D.Y., Lee, S.B., Tassinari T. and Macy, C., Biotechnol. Bioeng. 1982, 23, 1047-1067
15. Detroy, R.W., Lindenfelsen, L.A., Sommer, S. and Orton, W.L. Biotechnol. Bioeng. 1981, 23, 1527-1535
16. Buchholz, K., Puls. J., Godelmann, B. and Dietrichs, H.H., Process Biochem. 1981, Dec./Jan., 37-43
17. Ladisch, M.R., Ladisch, C.M. and Tsao, G.T., Science 1978, 201, 743-745
18. Reese, E.T., Process Biochem. 1982, May/June, 2-6
19. Castanon, M. and Wilke, C.R., Biotechnol. Bioeng. 1981, 23, 1365-1372

3 Enzymatic hydrolysis of beer brewers' spent grain and the influence of pretreatment

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ENZYMATIC HYDROLYSIS OF BEER BREWERS' SPENT GRAIN AND THE INFLUENCE OF PRETREATMENTS

G. Beldman, J. Hennekam and A.G.J. Voragen

Department of Food Science, Agricultural University, Wageningen

A batch of spent grain was found to contain 15.1% cellulose, 2.0% starch, 24.8% hemicellulose and 2.3% uronic acid on dry matter. For untreated spent grain, approximately 47% of these polysaccharides were hydrolysed by a mixture of two commercial cellulases.

Hammer milling and Ultra Turrax blending improved enzymatic hydrolysis by 10%; extrusion at 170°C in a single screw extruder yielded 18% more sugars.

Incubation for 4 hours at 90°C with 0.5 N NaOH or 0.5 N H_2SO_4 greatly improved enzymatic hydrolysis. About 80% of the polysaccharides were hydrolysed by a combination of chemical pretreatment and enzymatic hydrolysis. The character of the produced polysaccharide fragments varied considerably and was dependent on whether alkali or acid was used during chemical pretreatment.

INTRODUCTION

The enzymatic saccharification of plant material has been shown to be of interest in various fields, such as the production of fruit juices (1, 2) and the utilization of biomass (3). A combination of cellulase, pectinase and hemicellulases is usually used, because of the chemical composition of the matrix of plant cell walls.

For apples, beet pulp and potato fibre almost a complete hydrolysis of polysaccharides is obtained by combining cellulase and pectinase. For non-parenchymatic tissue the situation is somewhat different: pectin is a minor component and the hemicellulose content is much higher. Enzyme action is restricted by the lignin barrier and by the high crystallinity of cellulose in this material. For such materials mechanical, thermal or chemical pretreatments are necessary to achieve efficient hydrolysis (4, 5).

This paper describes various enzymatic treatments and chemical and physical pretreatment, using brewers' spent grain as substrate. Spent grain is the residue of malt and grain which remains in the mash-kettle after the liquefied and saccharified starch has been removed by filtration.

MATERIALS AND METHODS

Samples of spent grain were obtained from the Heineken Brewery at Zoeterwoude (the Netherlands). Sub-samples were freeze-dried and hammer milled to particles smaller than 0.5 mm (dried AsIs), in order to determine their composition. From other sub-samples, Alcohol Insoluble Solids (AIS) were prepared by 4 successive extractions with 96% ethanol, at 50°C and Water Insoluble Solids (WIS) by 4 successive extractions with distilled water at 50°C . These samples were also subjected to the same treatment and analysis of those used for dried AsIs.

Analysis methods

Moisture and ash were determined by standard methods. Cellulose was determined according to Updegraff (6), uronic acids with m-hydroxydiphenyl using the method of Ahmed-Labavitch (7), starch with an enzymatic test of Boehringer, (Boehringer, Mannheim, FRG), nitrogen according to Kjeldahl (8). Sugar composition was determined by GC-analysis of the alditolacetate derivates of the sugars (9) (glass column, 3 m x 2 mm i.d., of 3% OV 275 on Chromosorb WAW at 190°C) which were obtained by a modified Saeman hydrolysis (10) for 4 hours at 100°C in 2.0 N H_2SO_4.

Enzymatically or chemically solubilized sugars were assayed after centrifugation of insoluble solids, using the Nelson-Somogyi method for reducing end groups (11) and the Dubois method for total sugars (12). Glucose was used as standard.

The insoluble solid content after various treatments was determined by washing, centrifuging and freeze-drying the residues and expressed as percentage of the original amount of dry matter.

Soluble products were analysed by HPLC (Spectra Physics SP 8000, equiped with a Waters R401 refractive index detector), using the Aminex HPX 87P column (300 x 7.8 mm, Bio-Rad Labs, Richmond CA, USA). A mixed bed ion-exchanger, containing equal amounts of the ion-exchange resins AG 50W-X4 (H^+) and AG3-X4A (OH^-) (Bio-Rad Labs) was used as precolumn.

Pretreatments

Physical treatments applied were hammer milling (0.5 mm sieve), Ultra Turrax blending (for 5 minutes) and extrusion in a Battenfield single screw extruder at 170°C, using a speed of 45-105 rpm and a 1:3 screw compression ratio. For chemical treatments 2.5 g freeze-dried spent grain was suspended in 20 ml solutions with various concentrations of NaOH or H_2SO_4 (0.01-0.50 N), incubated at 90°C during 4 hours and subsequently neutralised to pH 4.85.

Enzymatic treatments

Several commercial enzyme preparations were tested for their ability to hydrolyse spent grain polysaccharides (Table I). Suspensions of spent grain (10% w/w, on dry weight) in 0.1 M succinate buffer pH 4.85 were incubated with enzymes for 70 hours at 40°C. To inhibit microbial growth 0.01% sodium azide and 0.2% sodium sulphite were added as preservatives. Physical pretreated samples were incubated under similar conditions, using 1% (w/w) Röhm cellulase, supplemented with 1% Röhm hemicellulase (after hammer milling and Ultra Turrax blending) or 1% Biocon Biocellulase supplemented with 1% Röhm cellulase (after extrusion). This latter combination of enzymes was also used for biodegradation of chemically pretreated samples, using the same reaction conditions.

Table I. Commercial enzyme preparations used for enzymatic hydrolysis of spent grain.

Enzymes	Supplier
Maxazyme CL 2000	Gist Brocades N.V., Delft, the Netherlands
Cellulase "2230A"	Röhm GmbH, Darmstadt, W-Germany
Hemicellulase "EL129-79"	" " "
Biocellulase A.C.	Biocon Ltd., Cork, Ireland
Cellobiase "SP188"	Novo Industri A/S, Bagsvaerd, Denmark
Pectinase K29	Rapidase, Seclin, France
Cellulase "9108"	" " "

RESULTS AND DISCUSSION

Composition of spent grain

The wet spent grain consisted of 20.4% dry matter, 19.6% WIS and 17.6% AIS. The compositions of the dried spent grain is

Table II. Composition of spent grain (g/100 g dry matter), after drying (AsIs), extraction with ethanol (AIS) and extraction with H_2O (WIS).

Constituent	AsIs	AIS	WIS
Ash	3.5	3.8	3.1
Protein	23.8	26.5	N.D.
Uronic Acids	2.3	2.5	2.5
Cellulose	15.1	17.9	16.9
Hemicellulose	24.8	30.4	31.1
Starch	2.0	2.3	1.7
Soluble Sugars	3.5	0.0	0.0
Fat + Lignin (by difference)	25.0	16.6	N.D.

N.D. = not determined

Table III. Sugar composition of spent grains (g/100 g dry matter), after 2.0 N H_2SO_4 hydrolysis. Spent grain samples were obtained after drying (AsIs), extraction with ethanol (AIS) and extraction with H_2O (WIS). Values are expressed as the amount of anhydro-sugar.

Anhydro-Sugar	AsIs	AIS	WIS
Rhamnose/Fucose	0.0	0.0	0.0
Arabinose	7.6	8.9	8.4
Xylose	15.5	19.8	20.0
Mannose	0.5	0.6	0.4
Galactose	1.2	1.2	1.2
Glucose	18.8	22.5	22.6

presented in Table II. Hemicellulose and cellulose are the main polysaccharides. The amount of pectic substances, expressed as uronic acids, was low. Starch was only present in minimal amounts. Table III shows the sugar compositions of the different samples after complete hydrolysis with 2 N H_2SO_4. If the sum of starch and cellulose content presented in Table II is compared with the amount of glucose found by GC-analysis (Table III), it can be seen that different methods of analysis gave very similar results.

Enzymatic hydrolysis of untreated spent grain

Table IV shows the solubilization and hydrolyis of polysaccharides after incubation of spent grain (AsIs) with several enzymes or combinations of enzymes. Differences in polysaccharide hydrolysis by the various enzyme preparations are small. Increasing the enzyme concentration from 1% to 2% (w/w,

Table IV. Amount of reducing sugars (RS) and total sugars (TS), released from spent grain by treatment with various enzyme preparations for 70 hours at 40°C and pH 4.85, as well as ratio of reducing sugars to total sugars and degree of polysaccharide hydrolysis.

Enzyme	RS	TS	RS/TS	polysaccharide hydrolysis **
	(mg/100 mg d.m.)			(%)
1%* Röhm cellulase	9.7	19.8	0.49	41
2% Röhm cellulase	10.4	21.2	0.49	44
1% Biocon Biocellulase	12.2	19.8	0.62	41
1% Maxazyme	8.4	15.8	0.53	33
1% Rapidase cellulase	11.2	20.9	0.54	44
1% Röhm cellulase +				
1% Röhm hemicellulase	12.0	21.0	0.57	44
1% Novo cellobiase	10.3	20.8	0.50	43
1% Biocon Biocellulase	12.3	22.4	0.55	47
1% Biocon Biocellulase +				
1% Röhm hemicellulase	13.1	22.3	0.59	46
1% Rapidase pectinase	13.7	19.8	0.69	41

* w/w based on dry matter
** Total sugars released, as % of polysaccharides present in spent grain (i.e. 48 mg/100 mg total solids).

on dry matter) resulted in only slightly more solubilized sugars. Mixing the cellulase with other enzyme preparations, including other cellulases (to overcome possible incomplete enzyme systems) had only minor effect. On the basis of the amount of total sugars, which were measured in the soluble fraction, 47% of the polysaccharides were hydrolysed by a mixture of Röhm cellulase and Biocon Biocellulase.

HPLC analysis showed that, besides of some oligomers, glucose was the main monomeric sugar found in the digest of this combination of enzymes (Fig. 1A). Re-incubation of the residue of enzyme treated spent grain (after removal of the soluble sugars) with fresh enzyme resulted in only minimal additional polysaccharide hydrolysis. Only 5% of the pentosans and 19% of the glucans of this residue were hydrolysed. These results indicate that the incomplete hydrolysis is mainly caused by the structural features of the unattacked cell wall fragments and not by product inhibition.

Physical pretreatment

Compared with untreated spent grain, both hammer milling and

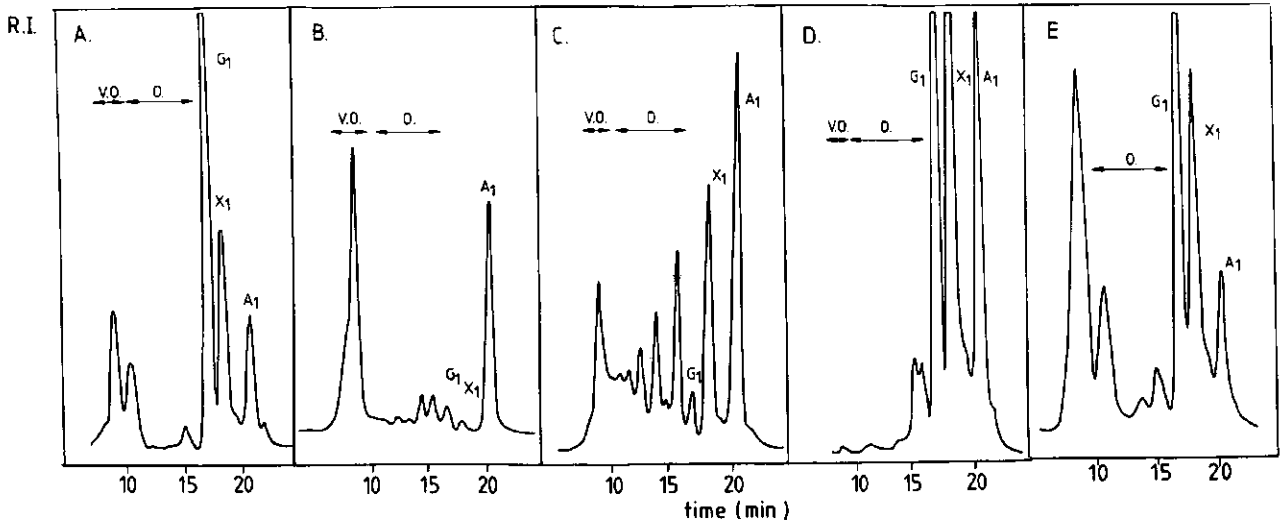

Fig. 1. HPLC analysis of products released from spent grain by cellulases (A), 0.1 N H_2SO_4 (B), 0.5 N H_2SO_4 (C), 0.5 N H_2SO_4 followed by cellulases (D) and 0.5 N NaOH followed by cellulases (E). The cellulase preparation consisted of a combination of Röhm cellulase and Biocon Biocellulase. A. arabinose, X_1, xylose, G_1, glucose, V.O., void oligomers, O, oligomer.

Ultra Turrax blending, which transform the macro-structure of spent grain to small fragments, yielded about 10% more total sugars after enzymic treatment. These sugars were derived equally from cellulose and hemicellulose hydrolysis. This indicates that the overall susceptibility to enzymatic hydrolysis was somewhat improved by these treatments which, however, did not affect the micro-structural features such as crystallinity and specific surface area of cellulose, known to have more influence on the degradibility (13, 14). The micro-structure can be affected by the efficient but expensive ball milling treatment, preferably with simultaneous enzymatic hydrolysis (15).

Extrusion resulted in 18% additional soluble sugars, mainly because cellulose hydrolysis was increased (results not shown).

Chemical pretreatment

As can be seen fom Table V, NaOH treatment resulted in the release of less soluble sugars from spent grain (35% for 0.5 N NaOH) than the H_2SO_4 treatment (66% for 0.5 N H_2SO_4). In comparison with the H_2SO_4 treatment, NaOH only slightly hydrolysed the extracted polysaccharides, resulting in a low ratio of reducing sugars to total sugars. Solubilization of non-sugar components was substantial in the sample treated with NaOH. From the material treated with 0.5 N NaOH only 38% of the original total solids remained as insoluble. However, at NaOH or H_2SO_4

Table V. Amount of reducing sugars (RS) to total sugars (TS) released from spent grain by NaOH and H_2SO_4 treatment, as wellas the ratio of reducing sugars to total sugars, amount of insoluble residual dry matter and degree of polysaccharide hydrolysis.

Treatment		RS	TS	Insoluble residue	RS/TS	Polysaccharide hydrolysis *
		(mg/100 mg d.m.)				(%)
H_2O		0.4	2.0	96.5	0.0	0
NaOH	0.50 N	1.5	17.0	38.4	0.09	35
	0.10 N	0.4	6.4	87.6	0.06	13
	0.01 N	0.4	2.0	94.2	0.20	4
H_2SO_4	0.50 N	14.6	31.0	59.2	0.46	66
	0.10 N	4.8	13.9	79.5	0.35	29
	0.01 N	0.6	2.9	89.0	0.21	6

* calculated as in Table IV

concentrations below 0.1 N scarcely any solubilization was effected, neither of sugar nor of non-sugar components.

HPLC-analysis of sugars obtained after H_2SO_4 treatment showed that arabinosyl linkages of spent grain are highly susceptible to acid hydrolysis, because at lower H_2SO_4 concentration mainly arabinose was released as monomer (Fig. 1B). At higher H_2SO_4 concentration next to arabinose also xylose and several oligomers were produced (Fig. 1C). Under these conditions almost no glucose was released. This suggests that cellulose was not hydrolysed by this acid treatment.

In the soluble fraction of the alkali treated material only larger oligomers and no monomers were found (results not shown).

Enzymatic hydrolysis of chemically pretreated samples

Enzymatic hydrolysis of NaOH pretreated spent grain resulted in an amount of solubilized total sugars, which was about equal to the amount of total sugars found in the digest of the H_2SO_4 pretreated material (Table VI). Compared with the results obtained when no treatment was applied, both chemical pretreatments doubled enzymatic hydrolysis of polysaccharides. However, the nature of the solubilized sugars differed considerably. The amount of reducing sugars obtained from the acid pretreated spent grain was much higher than the value found after enzymic conversion of the NaOH pretreated material, indicating that the hydrolysis products from the first treatment were almost completely in monomeric form.

Table VI. Amount of reducing sugars (RS) and total sugars (TS), released from chemically pretreated spent grain by a combination of Biocellulase and Röhm cellulase, as well as the ratio of reducing sugars to total sugars, amount of insoluble residual dry matter and degree of polysaccharide hydrolysis.

Treatment	RS	TS	Insoluble residue	RS/TS	Polysaccharide hydrolysis *
	(mg/100 mg d.m.)				(%)
H_2O + enzyme	12.4	22.0	76.6	0.56	46
NaOH 0.50N + enzyme	20.0	37.8	35.4	0.52	79
0.10N	15.8	26.8	61.5	0.59	56
0.01N	13.4	22.6	74.8	0.59	47
H_2SO_4 0.50N + enzyme	34.2	40.4	47.7	0.85	85
0.10N	20.2	28.0	68.0	0.72	58
0.01N	12.8	22.4	70.1	0.57	47

* calculated as in Table IV.

This was confirmed by HPLC analysis of the soluble products in both digests. Actually only the monomers glucose, xylose and arabinose and almost no oligomers were obtained as hydrolysis products of the acid pretreated sample (Fig. 1D). Essentially no cellobiose was detected. This implies that the combination of cellulases which were used are not deficient in cellobiase activity and possible product inhibition by cellobiose could be prevented. In the enzymic product of the 0.5 N NaOH pretreated material only 51% of the total peak area was found to be monomer and 29% as larger oligomers, appearing in the "void" of the chromatogram (Fig. 1E). It is not clear why these latter oligomers are not further hydrolysed by the enzymes to smaller products. It may be that these oligomers are heteroglycans which are only degraded by a special enzyme, for instance an arabinofuranosidase, lacking in the enzyme preparations which were used. Presumably these oligomers are (at least partially) hydrolysed during H_2SO_4 pretreatment and are therefore not detected in the enzyme digest of this material. The relatively small "void" peak in the chromatogram of the product of 0.5 N H_2SO_4 treatment alone (Fig. 1C) confirms this possibility.

REFERENCES

1. Voragen, A.G.J., Heutink, R. and Pilnik. W., J. Appl. Biochem. 1980, 2, 452-468
2. Voragen. A.G.J. and Pilnik. W., Flussiges Obst 1981, 48, 261-264
3. Beldman, G., Voragen, A.G.J., Rombouts, F.M. and Pilnik, W., Enzyme Microb. Technol. 1984, 6, 503-507
4. Fan, L.T., Lee, Y.H. and Beardmore, D.H., 2nd Int. Symposium on Bioconversion and Biochemical Engineering, Delhi, India, 3-6 March 1980
5. Gharpuray, M.M., Lee, Y.H. and Fan L.T., Biotechnol. Bioeng. 1983, 25, 157-172
6. Updegraff, D.M., Anal. Biochem., 1969, 32, 420-424
7. Ahmed, A.E.R. and Labavitch, J.M., J. Food Biochem. 1977, 1, 361-365
8. Roozen, J.P. and Ouwehand, R., Voedingsmiddelentechnol. 1978, 22, 1-3
9. Jones, T.M. and Albersheim, P., Plant Physiol. 1972, 49, 926-936
10. Selvendran, R.R., March, J.F., Ring, S.G., Anal. Biochem. 1979, 96, 282-292
11. Nelson, N., J. Biol. Chem. 1944, 153, 375-380
12. Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, F.A. and Smith, F., Anal. Chem. 1956, 28, 350-356

13. Lee, S.B., Shin, H.S., Ryu, D.D.Y. and Mandels, M., Biotechnol. Bioeng. 1982, 24, 2137-2153
14. Lee, S.B., Kim, I.H., Ryu, D.D.Y. and Taguchi, H., Biotechnol. Bioeng. 1983, 25, 33-51
15. Kelsey, R.G. and Shafizadeh, F., Biotechnol. Bioeng. 1980, 22, 1025-1037

4 The cellulase of *Trichoderma viride*

Purification, characterization and comparison of all detectable endoglucanases, exoglucanases and ß-glucosidases

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THE CELLULASE OF TRICHODERMA VIRIDE
Purification, characterization and comparison of all detectable endoglucanases, exoglucanases and β-glucosidases

G. Beldman, M.F. Searle-van Leeuwen, F.M. Rombouts and A.G.J. Voragen

Department of Food Science, Agricultural University, Wageningen

Six endoglucanases (Endo I; II; III; IV; V; VI), three exoglucanases (Exo I; II; III) and a β-glucosidase (β-gluc I) were isolated from a commercial cellulase preparation derived from Trichoderma viride, using gel filtration on Bio-Gel, anion exchange on DEAE-Bio-Gel A, cation exchange on SE-Sephadex and affinity chromatography on crystalline cellulose. Molecular masses were determined by polyacrylamide gel electrophoresis. One group of endoglucanases (Endo I, Endo II and Endo IV) with M_r of 50000, 45000 and 23500 were more random in their attack on carboxymethylcellulose than another group (Endo III, Endo V and Endo VI) showing M_r of 58000, 57000 and 53000 respectively. Endo III was identified as a new type of endoglucanase with relatively high activity on crystalline cellulose and moderate activtity on carboxymethylcellulose. Exo II and Exo III with M_r of 60500 and 62000 respectively showed distinct adsorption affinities on a column of crystalline cellulose and could be eluted by a pH gradient to alkaline regions. These enzymes were cellobiohydrolases as judged by high-pressure liquid chromatography of the products obtained from incubation with H_3PO_4-swollen cellulose. It was concluded that these exoglucanases are primarily active on newly generated chain ends. Exo I was essentially another type of exoglucanase which in the first instance was able to split off a cellobiose molecule from a chain end and then hydrolyse this molecule in a second step to two glucose units. β-Gluc I was a new type of aryl-β-D-glucosidase which had no activity on cellobiose. The enzyme had a M_r of 76000 and was moderately active on CM-cellulose, crystalline cellulose and xylan and highly active on p-nitrophenyl-β-D-glucose and p-nitrophenyl-β-D-xylose.

Abbreviations. HPLC, high-pressure liquid chromatography; SDS, sodium dodecyl sulphate.

Enzymes. 1,4-β-D-Glucan glucanohydrolase (EC 3.2.1.4); 1,4-β-D-glucan cellobiohydrolase (EC 3.2.1.91); β-D-glucoside glucohydrolase or β-glucosidase (EC 3.2.1.21); 1,4-β-D-glucan glucohydrolase (EC 3.2.1.74).

INTRODUCTION

Much attention is paid to the enzymatic hydrolysis of cellulose because it is a renewable carbon source and available in large quantities. Cellulases from fungal origin are known to be most powerful in cellulose hydrolysis. Cellulase preparations are derived, among other, from Trichoderma reesei (1, 2), Trichoderma viride (3), Trichoderma koningii (4) and Sporotrichum pulverulentum (5). These microorganisms produce a multicomponent enzyme system, including the 1,4-β-D-glucan glucanohydrolase (endoglucanase; EC 3.2.1.4), 1,4-β-D-glucan cellobiohydrolase (exoglucanase; EC 3.2.1.91) and β-D-glucoside glucohydrolase β-glucosidase; EC 3.2.21). A combination of these three types of enzymes is necessary for the complete hydrolysis of crystalline cellulose. Endoglucanase and exoglucanase are known to act synergistically in cellulose hydrolysis (6), while β-glucosidase is needed for removal of cellobiose, a strong inhibitor of both endoglucanase and exoglucanase (7). However, the debate on the mechanism of cellulose hydrolysis still continues. The diversity of endoglucanases, exoglucanases and β-glucosidases from fungal origin is emphasized by many review reports on this subject (7-13). Comparative studies are difficult and sometimes confusing since several species with different strains are used by various investigators and characterization is done using different criteria.

The first step in a study on the mode of action of cellulases is the isolation of substantial amounts of all enzymes of the complex. The purpose of the present work was the integral fractionation of a commercial cellulase preparation derived from Trichoderma viride and the characterization of all endoglucanases, exoglucanases and β-glucosidases which could be detected and purified, with respect to molecular mass, isoelectric point, glycoprotein nature, temperature optimum, degree of randomness in CM-cellulose hydrolysis and specific activity on several types of cellulose.

A new type of endoglucanase with relatively high activity on crystalline cellulose was isolated. We purified an aryl-β-D-

glucosidase, not reported before in the literature, which is able to act on p-nitrophenyl-β-D-glucoside but lacks the ability to hydrolyse cellobiose.

Affinity chromatography on crystalline cellulose was used for the separation of two different cellobiohydrolases. Hydrolysis products of these exoglucanases were analysed by HPLC and compared to the hydrolysis products of another type of exoglucanase, isolated from the same enzyme preparation. The mode of action of the cellulase complex, with respect to the role of these exoglucanases, is discussed.

MATERIALS AND METHODS

Enzyme preparation

Maxazyme Cl, a commercial cellulase preparation from Trichoderma viride origin, was kindly provided by Gist-Brocades (Delft, The Netherlands).

Enzyme assays

Enzyme activities were measured towards Avicel (type SF, Serva, Heidelberg, FRG; Avicelase activity), CM-cellulose (type Akucell AF 0305, Akzo, Arnhem, The Netherlands; CM-cellulase activity), p-nitrophenyl-β-D-glucopyranoside (Koch-Light, Colnbrook, Bucks, England; β-glucosidase activity), cellobiose (BDH Chemicals Ltd, Poole, England) and H_3PO_4-swollen cellulose which was obtained by the method described by Wood (14). All activities were expressed in International Units (U) ml^{-1}.

Avicelase activity. The reaction mixture for Avicelase activity measurement consisted of 0.5 ml 1% Avicel suspension in 0.05 M sodium acetate buffer, pH 5.0 and 0.5 ml of enzyme solution. Incubation took place in a shaking incubator at 30°C for 20 h. After centrifugation 0.5 ml was taken and analysed for reducing sugars by the method of Nelson Somogyi (15). Specific activites were measured at a protein concentration of 25 µg/ml.

CM-cellulase activity. CM-cellulase activity was measured in a mixture containing 0.4 ml of a 0.5% CM-cellulose solution in 0.05 M sodium acetate, pH 5.0 and 0.1 ml of enzyme solution. After incubation at 30°C for 1 h, the reaction was stopped and reducing sugars were measured by addition of the Nelson Somogyi reagent. Samples were centrifuged before reading absorbance at 520 nm. CM-cellulase activity was also measured viscometrically at 30°C according to Almin and Eriksson (16), using a 0.25% substrate concentration. Activity was expressed as rate of

change in specific fluidity, $\Delta\Phi_{sp}(min^{-1})$. A protein concentration of 1 µg/ml was used for specific activity measurements.

β-Glucosidase activity. The β-glucosidase assay was performed by addition of 0.1 ml of enzyme solution to 0.9 ml of 0.1% p-nitrophenyl-β-D-glucopyranoside in 0.05 M sodium acetate buffer, pH 5.0 and incubated for 1 h at 30°C. The reaction was stopped by addition of 1 ml 0.5 M glycine buffer, pH 9.0, containing 0.002 M EDTA. The concentration of p-nitrophenol was measured at 400 nm, using an absorption coefficient of 13700 $M^{-1}cm^{-1}$. Specific activities were measured using a protein concentration of 5 µg/ml.

Cellobiase activity. Enzyme fractions, containing β-glucosidase activity, were screened for cellobiase activity by incubation of 0.4 ml enzyme solution in 0.1 M sodium acetate pH 5.0, containing 20 µg protein with 0.1 ml 0.2% cellobiose in the same buffer. After incubation at 30°C for 1 h, the enzymes were inactivated by placing the tubes in a boiling water bath for 10 min. Glucose concentration was measured by the hexokinase method (Boehringer enzyme kit, Boehringer, Mannheim, FRG).

Activity towards H_3PO_4-swollen cellulose. Activity towards H_3PO_4-swollen cellulose was measured by addition of 0.1 ml enzyme solution to 0.9 ml 0.55% H_3PO_4-swollen cellulose in 0.05 M sodium acetate pH 5.0. Incubation took place for 20 h at 30°C. After centrifugation 0.5 ml of the supernatant was analysed for reducing sugars. Specific activities were measured at a protein concentration of 1 µg/ml.

Column chromatography

Column chromatography was carried out on Bio-Gel P10 (100-200 mesh), Bio-Gel P100 (100-200 mesh), DEAE-Bio-Gel A (Bio-Rad Laboratories, Richmond, USA) SE-Sephadex C50 (Pharmacia Fine Chemicals, Uppsala, Sweden) and Avicel crystalline cellulose. Eluted fractions were analysed for Avicelase, CM-cellulase and β-glucosidase acitivities. Protein was detected at 280 nm. Protein concentrations were measured using the Folin reagent (17) with bovine serum albumin as the standard. Chromatography experiments were carried out at 4°C. All buffers contained 0.01% NaN_3 as preserving agent. Details on the chromatographic steps are given under Results.

Sodium dodecyl suplhate/polyacrylamide-gel electrophoresis

Slab-gel electrophoresis on an 11% polyacrylamide gel, using the discontinuous buffer system of Laemmli (18) was applied to analyse enzyme purity. Samples were 3.5 times diluted with

sample buffer, consisting of 0.125 M Tris/HCl, pH 6.8, 2.5% (w/v) SDS, 2.5% (v/v) 2-mercaptoethanol, 25% (v/v) glycerol and 0.0025% bromphenol bleu and subsequently boiled for 2 min. Gels were stained for protein with Coomassie brilliant bleu and for carbohydrate, using the periodic acid/Schiff reagent (19). Bovine serum albumin (M_r 68000), ovalbumin (M_r 45000), and chymotrypsinogen (M_r 25000) were used as standards.

Isoelectric focusing

Analytical thin-layer gel isoelectric focusing was performed in the pH range 3.5-9.5 on te LKB 2117 Multiphor (LKB-Produkter AB, Stockholm, Sweden) using the application note provided by the manufacturer. The procedure was slightly modified: 0.4 ml 0.004% riboflavin was replaced by 0.4 ml 1% ammonium persulphate and 50µl N,N,N',N',-tetramethylethylethylendiamine (TEMED). After electrofocusing the gel was fixed with a solution containing 30% methanol/10% trichloroacetic acid/3.5% sulphosalicylic acid for 1 h and then with a solution containing 30% methanol/12% trichloroacetic acid for 2 h. Before staining all the ampholines were removed by washing with 40% methanol/10% acetic acid for at least 36 h.

Temperature optimum

Temperatre optima of all purified enzymes were determined by measuring the formation of reducing sugars from CM-cellulose at temperatures ranging from 10° to 90°C. Additionally, temperature optima of enzymes showing β-glucosidase activity were determined with p-nitrophenyl-β-D-glucopyranoside and enzymes showing mainly Avicelase activity were analysed with Avicel as substrate.

Product analysis on HPLC

In order to identify purified enzymes showing high Avicelase and low CM-cellulase activity, these proteins were incubated at a concentration of 10 µg/ml with 1% H_3PO_4-swollen cellulose at 30°C in 0.05 M sodium acetate pH 5.0, for 2, 6 and 21 h. After centrifugation of residual cellulose (10 min, 3000 x g) the enzymes in the supernatant were inactivated by placing the tubes in a boiling water bath for 5 min. Products were analysed by HPLC (Spectra Physics SP 8000) using an Aminex HPX 87P column and water as the eluent.

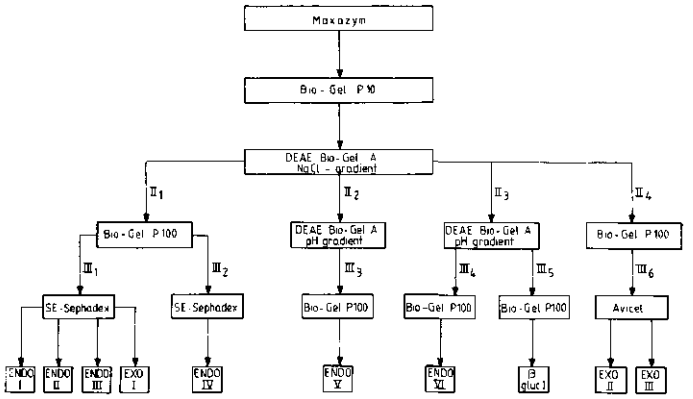

Fig. 1. Flow sheet of the purification of endoglucanases, exoglucanases and β-glucosidases from a commercial enzyme preparation.

RESULTS

Purification studies

The procedure which was applied for fractionation of the cellulase complex into its endoglucanase, exoglucanase and β-glucosidase components is shown in Fig. 1. An amount of 10 g cellulase preparation was dissolved in 30 ml 0.01 M sodium acetate, pH 5.0, centrifuged 20 min at 50000 x g to remove solids and desalted on a Bio-Gel P10 column (30 x 950 mm) equilibrated in the same buffer. All of the cellulase activity appeared as one peak in the void volume (results not shown). This peak was pooled and applied to a DEAE-Bio-Gel A column, equilibrated in 0.01 M sodium acetate pH 5.0. The column was washed with buffer and then eluted with a sodium chloride gradient in the same buffer (Fig. 2).

Peak II_1, containing most of the β-glucosidase and CM-cellulase activity was pooled, lyophilized, dissolved in a small volume and applied to a Bio-Gel P100 column, equilibrated in 0.02 M sodium citrate pH 3.5. After elution with the same buffer three peaks were obtained of which two peaks showed cellulase activity (Fig. 3).

Peak III_1 was dialysed and concentrated in 0.01 M sodium citrate pH 3.5 in an ultrafiltration cell (Diaflo type 50, Amicon, MA, USA) using the PM 10 membrane. This concentrated material was applied to a SE-Sephadex C50 column, equilibrated in 0.01 M sodium citrate pH 3.5. The column was eluted with a

flat and then a steep sodium citrate gradient (Fig. 4). Extensive purification of Endo II and Endo III was obtained after rechromatography on the same SE-Sephadex column. All the four peaks, Endo I, Endo II, Endo III and Exo I, containing cellulase and β-glucosidase activity, were dialysed and concen-

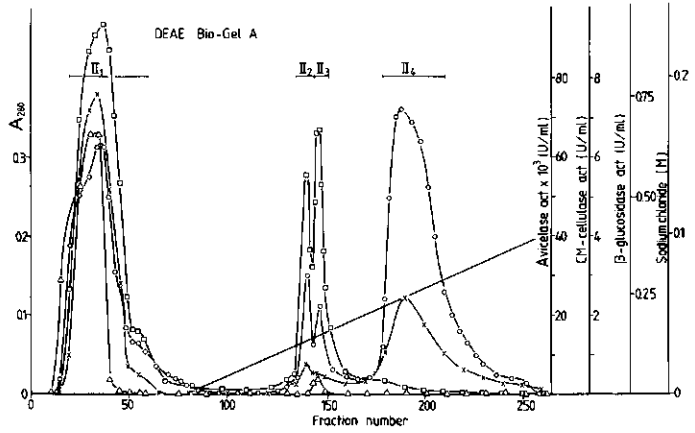

Fig. 2. DEAE-Bio-Gel A chromatography of crude cellulase preparation after desalting on Bio-Gel P10. Fractions from the Bio-Gel column containing cellulase activity were pooled (140 ml) and applied to the DEAE-Bio-Gel A column (30 x 200 mm). The column was washed with 300 ml 0.01 M sodium acetate pH 5.0 and then eluted with a linear gradient of sodium chloride in the same buffer. The flow rate was 25 ml/h and fraction volume was 5.5 ml. (○) Absorbance at 280 nm; (×) Avicelase; (□) CM-cellulase; (△) β-glucosidase; (—) sodium chloride concentration.

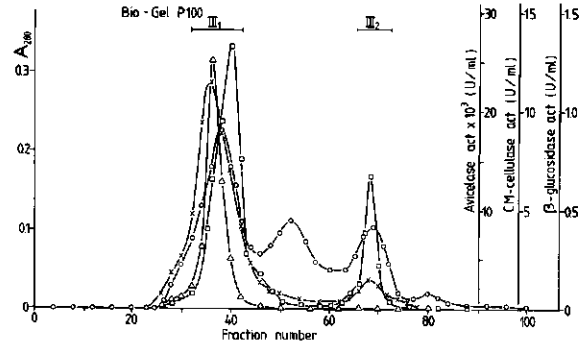

Fig. 3. Bio-Gel P100 chromatography of pool II_1. Pool II_1 from the DEAE-Bio-Gel A column was concentrated to a small volume and applied to the Bio-Gel P100 column (20 x 900 mm). The column was eluted with 0.02 M sodium citrate pH 3.5 with a flow rate of 25 ml/h. The fraction volume was 5 ml. (○) Absorbance at 280 nm; (×) Avicelase; (□) CM-cellulase; (△) β-glucosidase.

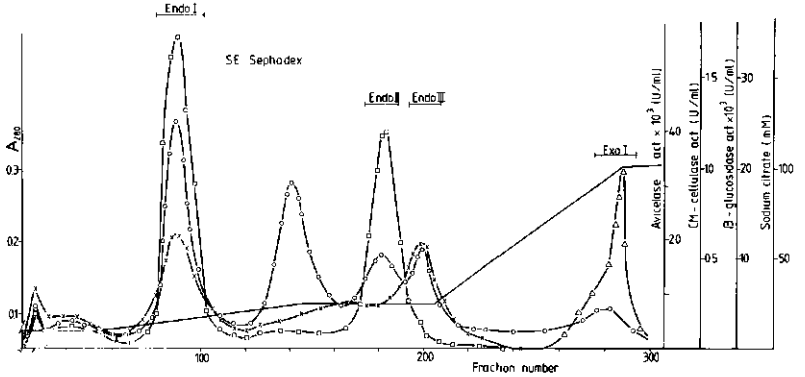

Fig. 4. SE-Sephadex chromatography of pool III_1. The dialysed and concentrated pool III_1 from the Bio-Gel P100 column was applied to the SE-Sephadex column (25 x 300 mm) equilibrated in 0.01 M sodium citrate, pH 3.5. The column was washed with 200 ml of the same buffer and then eluted with a linear gradient from 0.01 M to 0.025 M sodium citrate, pH 3.5. From fraction 150 to 205 the column was washed with 275 ml 0.025 M sodium citrate pH 3.5 and then elution was continued with a linear gradient to 0.1 M sodium citrate pH 3.5. The flow rate was 6 ml/h and the fraction volume was 5 ml. (○) Absorbance at 280 nm; (×) Avicelase; (□) CM-cellulase; (△) β-glucosidase; (—) sodium citrate concentration.

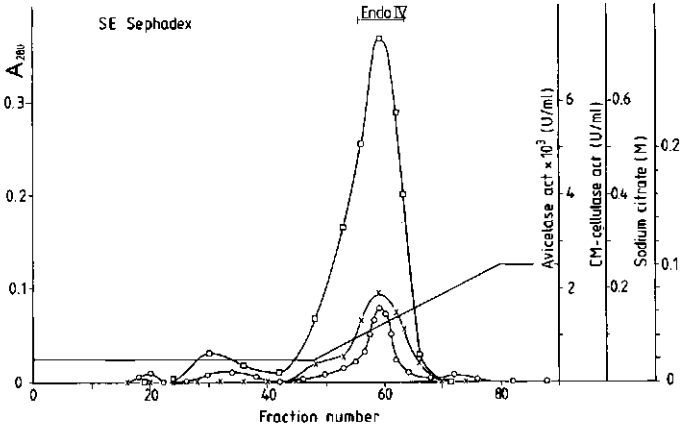

Fig. 5. SE-Sephadex chromatography of pool III_2. The concentrated pool III_2 from the Bio-Gel P100 column was applied to the SE-Sephadex column (20 x 200 mm) equilibrated in 0.02 M sodium citrate pH 3.5. The column was washed with 475 ml of the same buffer and then eluted with a linear gradient to 0.1 M sodium citrate pH 3.5. The flow rate was 20 ml/h and fraction volume was 10 ml. (○) Absorbance at 280 nm; (×) Avicelase; (□) CM-cellulase; (—) sodium citrate concentration.

trated by ultrafiltration in 0.05 M sodium acetate pH 5.0. These proteins appeared as a single band on SDS/polyacrylamide gel electrophoresis (Fig. 13). The first two peaks of Fig. 4, containing cellulase activity (Endo I and Endo II) were typical endoglucanases showing high specific activity towards CM-cellulose and low activity towards Avicel (Table 1).

The peak referred to as Endo III had low specific activity towards CM-cellulose and a much higher activity towards Avicel. This enzyme was able to produce cellotriose, cellobiose and glucose from H_3PO_4-swollen cellulose (Fig. 14d) and therefore identified as an endoglucanase. There was no evidence for cellotriose production from transglucosidase activity of this enzyme, because the relative peak areas of G_3, G_2 and G_1 remained constant in the course of the incubation (analysed after 2, 6 and 21 h; results not shown).

The last peak of the elution profile of Fig. 4 (Exo I) showed high aryl-β-glucosidase activity, and also high activity towards cellobiose, H_3PO_4-swollen cellulose and Avicel. HPLC analysis of the products obtained after incubation with H_3PO_4-swollen cellulose showed that this enzyme was an exoglucanase, releasing cellobiose molecules which were hydrolysed by the same

Table 1. Specific activities of endoglucanases, exoglucanases and β-glucosidase purified from commercial cellulase derived from Trichoderma viride.
Specific activities were measured at 30°C and pH 5.0 towards CM-cellulose, Avicel, H_3PO_4-swollen cellulose, p-nitrophenyl β-D-Glucopyranoside (pNp-Glc) and cellobiose as substrates. For experimental details see the text. Abbreviations: n.d., not detected; -, not determined.

Enzyme	Specific activity towards				
	CM-cellulose	Avicel	H_3PO_4-swollen cellulose	p-Np-Glc	cellobiose
	U/mg				
Endo I	13.1	0.0130	0.64	n.d.	-
Endo II	20.1	0.0068	0.46	n.d.	-
Endo III	3.2	0.0156	0.45	n.d.	-
Endo IV	9.6	0.0026	0.33	n.d.	-
Endo V	14.7	0.0073	0.61	n.d.	-
Endo VI	15.8	0.0044	0.36	n.d.	-
Exo I	1.8	0.0160	0.63	0.79	0.19
Exo II	0.26	0.0064	0.053	n.d.	-
Exo III	0.033	0.0078	0.030	n.d.	-
β-Gluc I	0.29	0.0018	0.45	0.40	n.d.

enzyme to two glucose units (Fig. 14a, b, c).

Pool III_2 from the Bio-Gel P100 column (Fig. 3) was concentrated by ultrafiltration and applied to a SE-Sephadex column (Fig. 5). The column was washed with 0.02 M sodium citrate and eluted with a sodium citrate gradient. The peak containing CM-cellulase activity was pooled and dialysed and concentrated in 0.05 M sodium acetate pH 5.0 by ultrafiltration. The protein was pure as judged by SDS/polyacrylamide gel electrophoresis (Fig. 13). This enzyme was identified as a typical endoglucanase (Endo IV), having high activity towards CM-cellulose and low activity towards Avicel (Table 1).

Peak II_2 from the DEAE-Bio-Gel A column (Fig. 2.) was rechromatographed on the same column, but this time using a pH gradient (Fig. 6). The peak III_3, containing CM-cellulase activity, was concentrated by lyophilization and applied to a Bio-Gel P100 column, equilibrated in 0.05 M sodium citrate pH 4.0 (Fig. 7). The peak containing CM-cellulase activity (Endo V) was dialysed and concentrated in 0.05 M sodium acetate, pH 5.0 by ultrafiltration. SDS/polyacrylamide gel electrophoresis of this protein showed a single band (Fig. 13). The enzyme was identified as an endoglucanase, with high activity towards CM-cellulose and low activity towards Avicel (Table 1).

The same pH gradient purification step on DEAE-Bio-Gel A was used for further purification of proteins in pool II_3 from the

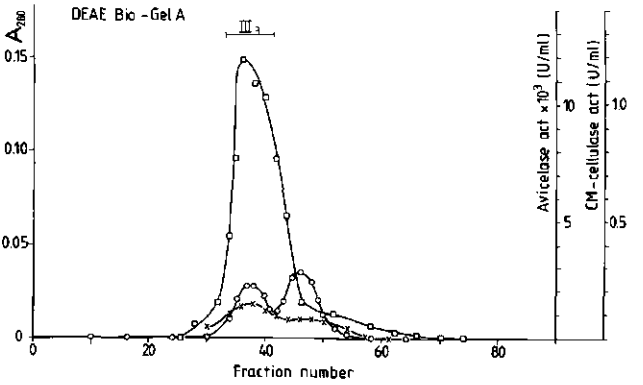

Fig. 6. DEAE-Bio-Gel A chromatography of pool II_2. Pool II_2 from the first DEAE-Bio-Gel A column was diluted with water to the same conductivity as the starting buffer (0.02 M sodium acetate pH 5.6) and applied to the DEAE-Bio-Gel A column (30 x 200 mm) equilibrated in the same buffer. The column was eluted with a pH gradient in 0.02 M sodium acetate from pH 5.6 to pH 3.8. The flow rate was 25 ml/h and fraction volume was 10 ml. (o) Absorbance at 280 nm; (x) Avicelase; (□) CM-cellulase.

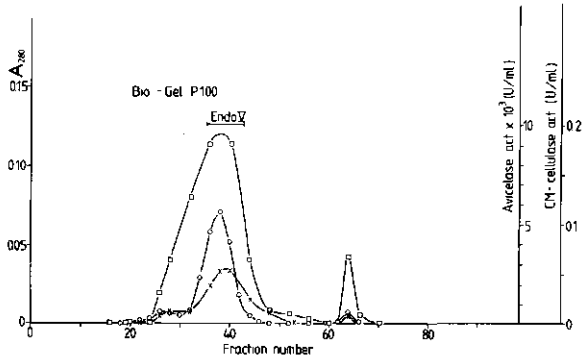

Fig. 7. Bio-Gel P100 chromatography of pool III_3. The lyophilized pool III_3 from the DEAE-Bio-Gel A column was dissolved in a small volume and applied to the Bio-Gel P100 column (20 x 900 mm) equilibrated in 0.05 M sodium citrate pH 4.0. The column was eluted with the same buffer. The flow rate was 25 ml/h and fraction volume was 5 ml. (○) Absorbance at 280 nm; (×) Avicelase; (□) CM-cellulase.

first DEAE-Bio-Gel column. Two CM-cellulase peaks and one β-glucosidase peak were obtained after this purification step (Fig. 8). The fist peak was identified as the same material as fraction III_3 (Fig. 6; results not shown). Bio-Gel P100 chromatography was used for further purification of pool III_4, containing CM-cellulase activity (Fig. 9), and pool III_5, containing β-glucosidase activity (Fig. 10).

The peak in Fig. 9 containing CM-cellulase activity (Endo VI) was concentrated by ultrafiltration and identified as a pure endoglucanase. A part of the CM-cellulase activity of Endo VI appeared as a second peak in the purification of the β-glucosidase (Fig. 10). This protein was identified as pure Endo VI.

The β-glucosidase peak of Fig. 10 (β-gluc I) consisted of a pure enzyme (Fig. 13) with high activity towards p-nitrophenyl-β-D-glucopyranoside. However, this enzyme showed no activity towards cellobiose and only moderate activity against Avicel, H_3PO_4-swollen cellulose and CM-cellulose (Table 1). The enzyme was also active towards xylan and the p-nitrophenyl derivatives of β-D-xylose and α-L-arabinose (results not shown).

Peak II_4 from the first fractionation on the DEAE-Bio-Gel A column (Fig. 2), containing essentially only Avicelase activity was concentrated by lyophilization and applied to the Bio-Gel P100 column (Fig. 11). The peak III_6, containing most of the Avicelase activity was subjected to affinity chromatography on

Avicel cellulose. Two peaks (Exo II and Exo III), containing Avicelase activity, were eluted from the column by a pH gradient of 0.1 M potassium phosphate, from pH 6.0 to pH 11.8 (Fig. 12). The fractions containing protein were immediately neutralized to pH 7 by addition of 2.5 ml 0.2 M KH_2PO_4, dialyzed and con-

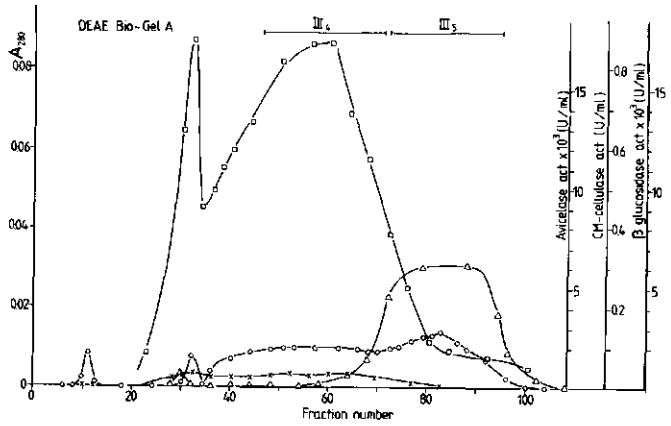

Fig. 8. DEAE-Bio-Gel A chromatography of pool II_3. Pool II_3 from the first DEAE-Bio-Gel A column was diluted with water to the same conductivity as the starting buffer, 0.02 M sodium acetate pH 5.6, and applied to the DEAE-Bio-Gel A column (30 x 200 mm) equilibrated in the same buffer. The column was eluted with a pH gradient in 0.02 M sodium acetate from pH 5.6 to pH 3.8. The flow rate was 25 ml/h and fraction volume was 10 ml. (o) Absorbance at 280 nm; (x) Avicelase; (□) CM-cellulase; (Δ) β-glucosidase.

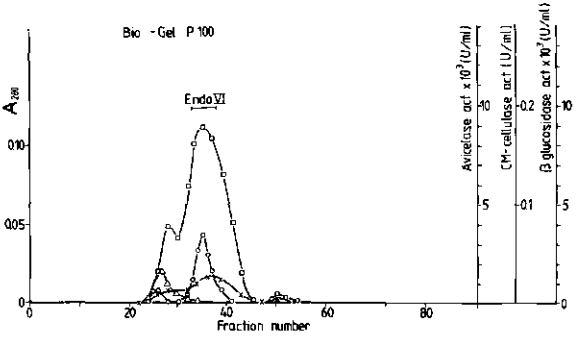

Fig. 9. Bio-Gel P100 chromatography of pool III_4. The lyophilized pool III_4 from the DEAE-Bio-Gel A column was dissolved in a small volume and applied to the Bio-Gel P100 column (20 x 900 mm) equilibrated in 0.05 M sodium citrate pH 4.0. The column was eluted with the same buffer. The flow rate was 25 ml/h and fraction volume was 5 ml. (o) Absorbance at 280 nm; (x) Avicelase; (□) CM-cellulase; (Δ) β-glucosidase.

centrated in 0.05 M sodium acetate by ultrafiltration. Exo II was eluted from the column at pH 10.3 and Exo III at pH 11.8. Both Exo II and Exo III gave single bands on gel electrophoresis. These enzymes were identified as exoglucanases, having high specific activity towards Avicel and very low activity towards

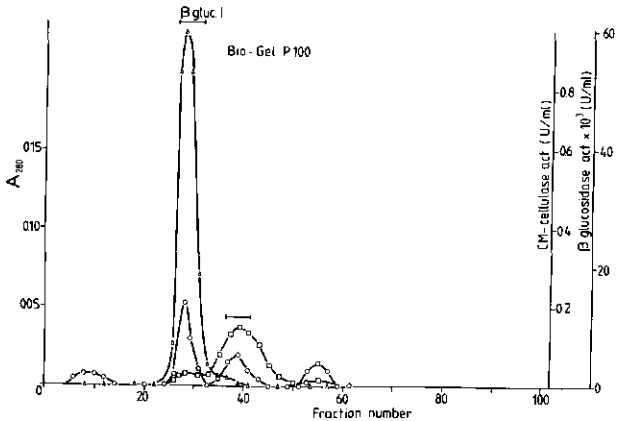

Fig. 10. Bio-Gel P100 chromatography of pool III_5. The lyophilized pool III_5 from the DEAE-Bio-Gel A column was dissolved in a small volume and applied to the Bio-Gel P100 column (20 x 900 mm) equilibrated in 0.05 M sodium citrate pH 4.0. The column was eluted with the same buffer. The flow rate was 25 ml/h and fraction volume was 5 ml. (○) Absorbance at 280 nm; (□) CM-cellulase; (△) β-glucosidase.

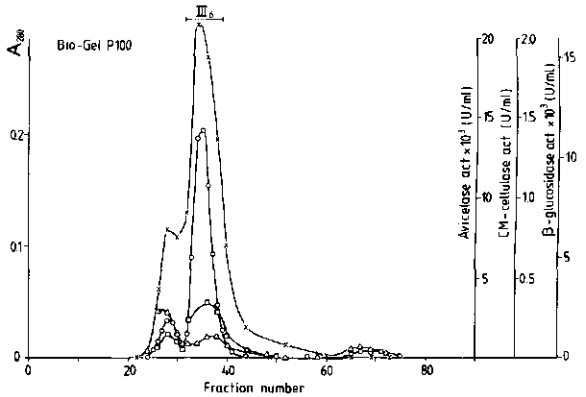

Fig. 11. Bio-Gel P100 chromatography of pool II_4. The lyophilized pool II_4 from the first DEAE-Bio-Gel A column was dissolved in a small volume and applied to the Bio-Gel P100 column (20 x 900 mm) equilibrated in 0.05 M sodium citrate pH 4.0. The column was eluted with the same buffer. The flow rate was 25 ml/h and fraction volume was 5 ml. (○) Absorbance at 280 nm; (×) Avicelase; (□) CM-cellulase; (△) β-glucosidase.

CM-cellulose and H_3PO_4-swollen cellulose (Table 1). These enzymes released cellobiose as the only product during incubation with H_3PO_4-swollen cellulose (Fig. 14e, f).

Degree of randomness of endoglucanases

The ratio of the activity of endoglucanases on CM-cellulose, measured viscometrically, to the activity on CM-cellulose, measured by the increase of reducing sugars (i.e.$\Delta\ \Phi_{sp}$/reducing

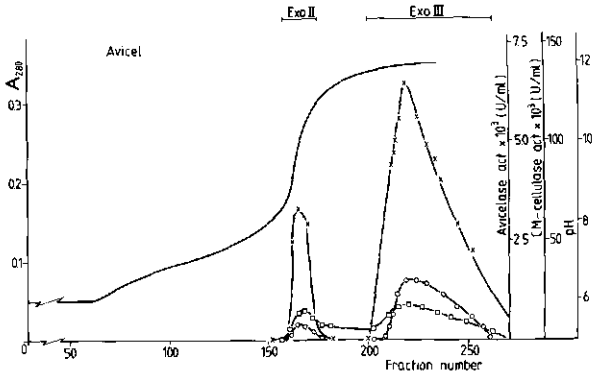

Fig. 12. Avicel chromatography of pool III_6. Pool III_6 was dialysed by ultrafiltration to 0.1 M potassium/sodium phosphate pH 6.0 and applied to the Avicel colum (22 x 100 mm), equilibrated in the same buffer. The column was washed with 500 ml buffer and then eluted with a pH gradient in 0.1 M potassium/-sodium phosphate from pH 6.0 to pH 11.8. The flow rate was 50 ml/h and fraction volume was 5 ml. (O) Absorbance at 280 nm; (X) Avicelase; (□) CM-cellulase.

Table 2. Degree of randomness in attack on CM-cellulose of endoglucanases isolated from Trichoderma viride.
The specific activity on CM-cellulose of the endoglucanases, measured viscometrically, was divided by the activity found from measurements of reducing power.

Enzyme	$\Delta\Phi_{sp}$/reducing power
Endo I	1.00
Endo II	0.77
Endo III	0.40
Endo IV	0.88
Endo V	0.50
Endo VI	0.49

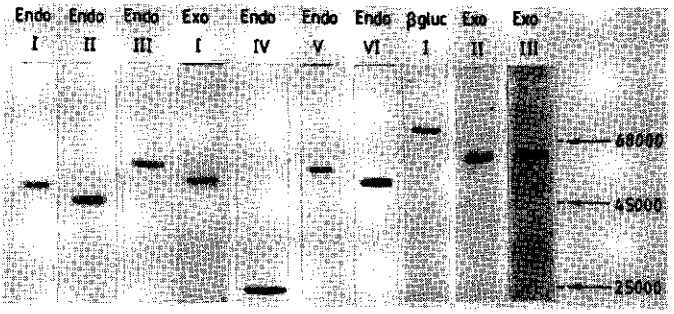

Fig. 13. SDS/polyacrylamide gel electrophoresis of endoglucanases, exoglucanases and β-glucosidase purified from commercial cellulase derived from Trichoderma viride. The gel had a final acrylamide concentration of 11%. Proteins were stained with Coomassie brilliant blue. Standards: bovine serum albumin (M_r 68000), ovalbumin (M_r 45000) and chymotrypsinogen (M_r 25000).

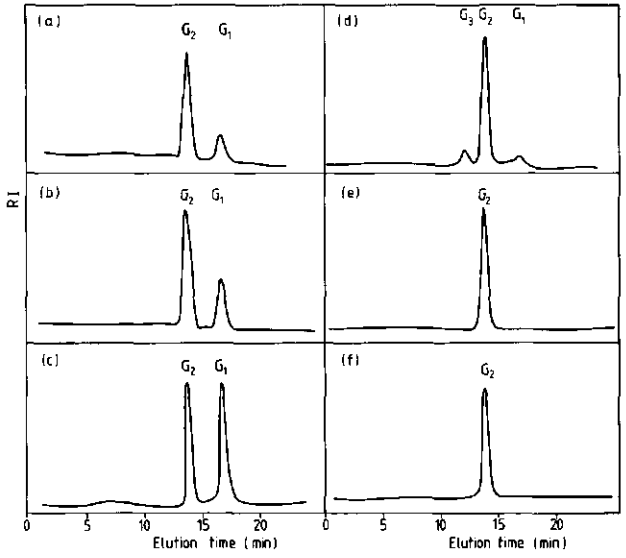

Fig. 14. HPLC analysis of products released from H_3PO_4-swollen cellulose. Incubation took place in 0.05 M sodium acetate pH 5.0 at 30°C with: Exo I for 2 h (a), 6 h(b), 21 h (c); Endo III for 21 h (d); Exo II for 21 h (e); Exo III for 21 h (f). G_1, glucose; G_2, cellobiose; G_3, cellotriose; RI, refractive index.

power), is an indication of the degree of randomness in cellulose hydrolysis. Essentially two types of endoglucanases could be distinguished (Table 2). Endo I, Endo II and Endo IV gave a high quotient and therefore were identified as randomly acting endoglucanases. Endo III, Endo V and Endo VI were a less-random type endoglucanases.

Chemical and physical properties

Molecular masses of the purified endoglucanases, exoglucanases and β-glucosidases could be estimated from the results of the SDS/polyacrylamide gel electrophoresis (Fig. 13). Most of the purified enzymes had M_r in the range 45000 to 76000 (Table 3). Endo IV appeared to be an exceptionally small protein. The special nature of this enzyme was emphasized by its relatively high isoelectric point. Moreover, Endo IV was the only enzyme of all the purified enzymes presented in this paper which could not be stained by the periodic acid/Schiff reagent. All the other endoglucanases, exoglucanases and β-glucosidases were identified as glycoproteins.

The glucanases Endo I, Endo II and Endo III and the glucanase Exo I, which were obtained by purification on the first SE-Sephadex column and which therefore had a relatively high isoelectric point (pI 5.3-6.9), showed a temperature optimum for hydrolysis of CM-cellulose at 60°C. This temperatre optimum was about 10°C higher than the optimal temperature of all the other endoglucanases, exoglucanases and β-glucosidases, which were isolated in the other purification steps and have a lower isoelectric point (pI 2.8 - 4.4). Again there is an exception for Endo IV, which had a pI of 7.7 and a temperature optimum of 48°C. The temperature optima of the enzymes showing β-glucosidase activity were also measured on p-nitrophenyl β-D-glucoside. There was no significant difference of optimal temperature when this substrate was used instead of CM-cellulose. Replacement of CM-cellulose by Avicel had almost no influence on the temperature optima of Exo II and Exo III, although the incubation time with the latter substrate was 20 h instead of 1 h with CM-cellulose. Although there is no explanation for the correlation between high temperature optima and high isoelectric points, the endoglucanase isolated from the thermophilic bacterium Clostridium thermocellum also has the combination of these two properties (Ng et al. [20]).

Table 3. Chemical and physical properties of endoglucanases, exoglucanases and β-glucosidase purified from commercial cellulase derived from Trichoderma viride.
For experimental details see the text. Abbreviations: P, positive, and N, negative on staining with periodic acid/Schiff reagent; -, not determined; pI, isoelectric point; pNp-Glc, p-nitrophenyl β-D-glucoside.

Enzyme	M_r	pI	Temp. optimum towards			Glycoprotein
			CM-cellulose	Avicel	pNp-Glc	
			°C			
Endo I	50000	5.3	60	-	-	P
Endo II	45000	6.9	60	-	-	P
Endo III	58500	6.5	60	-	-	P
Endo IV	23500	7.7	48	-	-	N
Endo V	57000	4.4	50	-	-	P
Endo IV	52000	3.5	50	-	-	P
Exo I	53000	5.3	60	-	62	P
Exo II	60500	3.5	52	50	-	P
Exo III	62000	3.8	52	50	-	P
β-Gluc I	76000	3.9	52	-	49	P

DISCUSSION

Endo I, Endo II, Endo IV, Endo V and Endo VI were identified as typical endoglucanases (1,4-β-D-Glucan glucanohydrolases), showing high activity towards CM-cellulose. The specific activities of these enzymes on Avicel were at least 10^3 times lower These two properties are generally accepted to distinguish endo-

glucanases from exoglucanases. Schoemaker et al.[21,22] describe a low-molecular-mass endoglucanase II from Pancellase SS, a commercial cellulase from Trichoderma viride, which clearly resembles Endo IV with respect to molecular mass and low carbohydrate content. The same enzyme was also purified by Fagerstam et al. [23] from T. viride QM 9414 and identified as a protein with a high isoelectric point (pI 7.5), which is consistent with our observations.

Schoemaker reports two other endoglucanases with different degrees of randomness in their action on CM-cellulose: endoglucanase III and endoglucanase IV [21, 22]. The less-random type of enzyme, referred to by these authors as endoglucanase III was very similar to Endo V, reported in this paper. Endo V is a less-random type of hydrolase than Endo I since it gave less decrease of viscosity compared to the increase of reducing sugars during incubation with CM-cellulose. For this reason and because it has the same molecular mass, Endo I was identified as the same endoglucanase (endoglucanase IV), isolated by Schoemaker [21, 22].

Endo VI is a glucanase with the same properties as Endo V, with respect to temperature optimum, carbohydrate content and degree of randomness in hydrolysis of CM-cellulose. Its molecular mass is somewhat lower. Endo VI was not described by Schoemaker [21, 22]. However, Okada [24] purified a less-random type of endoglucanase (cellulase II-b) from Meicellase, a commercial cellulase from T. viride which is similar to Endo VI.

Until now three different types of β-glucosidase isolated from T. viride have been reported. Two of these enzymes are extracellular and have M_r of 47000 [25] and 76000 [26] respectively. Another, intracellular β-glucosidase with a M_r of 98000, was isolated by Inglin et al. [27]. All of these enzymes showed aryl-β-D-glucosidase as well as cellobiase activity. Here we describe a new type of β-glucosidase (β-gluc I) which is active towards p-nitrophenyl β-D-glucoside but is unable to hydrolyse cellobiose. The enzyme showed a broad substrate specifcity: it was moderately active towards CM-cellulose, Avicel and xylan and highly active towards the p-nitrophenyl derivatives of β-D-glucose and β-D-xylose.

Fägerstam et al. [23] isolated three cellobiohydrolases: a major component with a pI of 3.95 and two minor components with pI 3.80 and 4.15 respectively. Exo III, described in this paper, resembles the major exoglucanase component, while Exo II is comparable to the minor component of pI 3.80. Exo II and Exo III

showed different adsorption affinities to crystalline cellulose and could easily be separated by specific desorption with a pH gradient without significant loss of enzyme activity. The diversity of the cellobiohydrolases was confirmed by Gum et al. [28], who isolated three exoglucanases from Meicelase P and one from T. viride QM 9123. These authors concluded that the carbohydrate content is the principal factor which differentiates the cellobiohydrolase enzymes. However, Nakayama et al. [29] concluded that limited proteolysis may partially be responsible for the multiplicity of endoglucanases from T. viride. Our results gave no evidence for any significant proteolysis during enzyme purification: although the period of time between two subsequent chromatographic steps in separate isolation experiments was varied, no distinct differences in elution profiles were obtained.

Exo II and especially Exo III acted synergistically with the endoglucanases described in this paper (results to be published; Chapter 6). A new type of synergism was demonstrated by Fägerstam et al.[30]. Their results show synergistic action between the cellobiohydrolase (CBH I) described in [23] and another cellobiohydrolase (CBH II), active against crystalline cellulose and which could be resolved into two iso-enzymes with pI values of 5.0 and 5.6 respectively. These enzymes produce cellobiose as the main product when acting on cystalline cellulose. However, significant amounts of glucose were also measured. Although these authors give no further information on molecular masses and specific activities towards the substrates which were used in this work, the results strongly suggest that the CBH II is not only a cellobiohydrolase but an exoglucanase identical to Exo I, described in this paper. Exo I is an enzyme with the same pI value and produces not only cellobiose from cellulose but also glucose since this enzyme has cellobiase activity as well. Moreover, the exoglucanase showed a strong synergistic action with Exo II and Exo III (result to be published; Chapter 6). Similar exoglucanases were isolated by Shikata et al. [31] from Cellulase-Onozuka derived from T. viride and by Wood et al. [32] from Penicillium funiculosum. The latter found little accumulation of cellobiose after an 18-h incubation with H_3PO_4-swollen cellulose and identified this enzyme as a 1,4-β-D-glucan glucohydrolase (EC 3.2.1.74).

Endo III showed low activity towards CM-cellulose but from all purified endoglucanases this enzyme had the highest specific activity against Avicel. An enzyme with these properties is

usually called an exoglucanase. However, from several lines of evidence presented in this paper we suggest that this enzyme is clearly an endoglucanase. Firstly, because Endo III released cellotriose, cellobiose and glucose from H_3PO_4-swollen cellulose, which is an indication for its endo-type attack. Secondly, this endoglucanase showed a synergistic action in combination with Exo III. Thirdly, no synergism was observed when Endo III was working in concert with an other endoglucanase (results to be published; Chapter 6). As far as we know, this enzyme has not been described before in the literature, and a further characterization will be published in a subsequent paper.

Comparison of the three purified exoglucanases with respect to their activities towards several substrates shows some interesting phenomena. The activity of Exo I against Avicel cellulose is about twice as high, compared to the activities of Exo II and Exo III, towards this substrate. However, using H_3PO_4 -swollen cellulose as the substrate Exo I showed 10-20 times more activity than Exo II and Exo III. These two cellobiohydrolases are not able to degrade this soluble, non-crystalline cellulose although there are sufficient chain ends available, since Exo I is highly active towards this substrate.

Exo II and Exo III have apparently a much higher affinity towards crystalline cellulose than towards amorphous cellulose (CM-cellulose; H_3PO_4-swollen cellulose). The low affinity towards these substrates is neither determined by the availability of chain ends, where Exo II and Exo III are supposed to bind and exert their catalytic action, nor by charge interactions between enzyme and substrate, because endoglucanases with different isoelectric points (in the range from pI 3.5 to 6.9) all show high affinity to these substrates under the experimental conditions (pH 5.0; 30°C). Why do Exo II and Exo III only have such high affinity for crystalline areas, while in these very regions the enzymes are unable to hydrolyse? One explanation could be that Exo II and Exo III also have a non-hydrolytic activity during degradation of cellulose: the induction of structural changes in the crystalline regions, resulting in better accessibility of the cellulose chains to endoglucanases. This is the well-known C_1, C_x concept of Reese[33], recently supported by the electron microscopic study of Chanzy et al.[34]

Another explanation, which is more in accordance with the hydrolytic nature of Exo II and Exo III, could be that these cellobiohydrolases adsorb on the crystalline regions and only

become active when an endoglucanase generates a new chain end. The results suggest that the cellobiohydrolase is especially active on such newly generated chain ends, since substrates with accessible chain ends (CM-cellulose, H_3PO_4-swollen cellulose) are scarcely attacked. This means that this enzyme would have to work in very close cooperation with the endoglucanase, maybe as an enzyme-enzyme complex on the surface of the cellulose chains, as suggested by Wood [6]. A detailed study on the adsorption kinetics of these exoglucanases and endoglucanases will give more insight into this theory and will be published in a subsequent paper.

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REFERENCES

1. Mandels, M., Meideiros, J.E., Andreotti, R.E. & Bisset, F.H. Biotechnol. Bioeng. 1981, 23, 2009-2026
2. Tangu, S.K., Blanch, H.W. & Wilke, C.R., Biotechnol. Bioeng. 1981, 23, 1837-1849.
3. Shin, S.B. Kitakawa, Y., Suga, K.I. & Ichikawa, K., J. Ferment. Technol. 1978, 56, 396-402
4. Wood, T.M., Biochem. J. 1968, 109, 217-227
5. Eriksson, K.E. & Pettersson, B., Eur. J. Biochem. 1975, 51, 193-206
6. Wood, T.M. & McCrae, S.I., Biochem. J. 1978, 171, 61-72
7. Woodward, J. & Wiseman, A., Enzyme Microb. Technol. 1982, 4, 73-79
8. Ladisch, M.R., Lin, K.W., Voloch, M. & Tsao, G.T., Enzyme Microb. Technol. 1983, 5, 82-102
9. Goksoyr, J. & Eriksen, J. in Microbial Enzymes and Bioconversions (Rose, A.H., ed.) 1980, pp. 283-329, Academic Press, New York
10. Ruy, D.D.Y. & Mandels, M., Enzyme Microb. Technol. 1980, 2, 91-102
11. Lee, Y.H. & Fan, L.T., Adv. Biochem. Eng. 1980, 17, 101-129
12. Bisaria, V.S. & Ghose, T.K., Enzyme Microb. Technol. 1981, 3, 90-104
13. Shewale, J.G., Int. J. Biochem. 1982, 14, 435-443
14. Wood, T.M., Biochem J. 1971, 121, 353-362
15. Somogyi, M., J. Biol. Chem. 1952, 195, 19-23
16. Almin, K.E. & Eriksson, K.E., Biochim. Biophys. Acta, 1967, 139, 238-247
17. Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J., J. Biol. Chem. 1951, 193, 265-275
18. Laemmli, U.K., Nature (Lond.) 1970, 227, 680-685
19. Colowick, S.P., Kaplan, N.D., Methods Enzymol. 1972, 28, 54-62
20. Ng, T.K. & Zeikus, J.G., Biochem. J. 1981, 199, 341-350
21. Schoemaker, S.P. & Brown, R.D., Biochim. Biophys. Acta,1978, 523, 133-146
22. Schoemaker, S.P. & Brown, R.D., Biochim. Biophys. Acta,1978, 523, 147-161

23. Fägerstam, L., Hakansson, U., Pettersson, G. & Andersson, L. Proc. Bioconversion Symp. IIT Delhi, 1972, 165-178
24. Okada, G., J. Biochem (Tokyo) 1975, 77, 33-42
25. Berghem, L.E.R. & Pettersson, L.G., Eur. J. Biochem. 1974, 46, 295-305
26. Gong, C.S., Ladisch, M.R. & Tsao, G.T., Biotechnol. Bioeng. 1977, 19, 959-981
27. Inglin, M., Feinberg, B.A. & Loewenberg, J.R., Biochem. J. 1980, 185, 515-519
28. Gum, E.K. & Brown, R.D., Biochim. Biophys. Acta 1977, 492, 225-231
29. Nakayama, M., Tomita, Y., Suzuki, H. & Nisizawa, K., J. Biochem. (Tokyo) 1976, 79, 955-966
30. Fägerstam, L.G. & Pettersson, L.G., FEBS Lett. 1980, 153, 113-118
31. Shikita, S. & Nisizawa, K., J. Biochem. (Tokyo) 1975, 78, 499-512
32. Wood, T.M. & McCrae, S.I., Carbohydr. Res. 1982, 110, 291-303
33. Reese, E.T., Rec. Adv. Phytochem. 1976, 11, 311-367
34. Chanzy, H., Henrissat, B., Vuong, R. & Chulein, M., FEBS Lett. 1983, 153, 113-118

5 Adsorption and kinetic behaviours of purified endoglucanases and exoglucanases from *Trichoderma viride*

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ADSORPTION AND KINETIC BEHAVIOURS OF PURIFIED ENDOGLUCANASES AND EXOGLUCANASES FROM TRICHODERMA VIRIDE.

G. Beldman, A.G.J. Voragen, F.M. Rombouts, M.F. Searle-van Leeuwen and W. Pilnik.

Department of Food Science, Agricultural University, Wageningen

Adsorption on crystalline cellulose of six endoglucanases (Endo I, II, III, IV, V and VI; 1,4-β-D-glucan glucanohydrolase, EC 3.2.1.4) and two exoglucanases (Exo II and III; 1,4-β-D-glucan cellobiohydrolase, EC 3.2.1.92), purified from a commercial cellulase preparation of Trichoderma viride origin, was studied. Endo I, III and V adsorbed strongly on Avicel cellulose, while adsorption of Endo II, IV and VI was much lower. Also, the two exoglucanases could be divided into one enzyme (Exo III) which had a high adsorption affinity and another enzyme (Exo II) which adsorbed only moderately. Adsorption data fitted the Langmuir-type adsorption isotherm. However, adsorption was only partially reversible with respect to dilution. No relation could be found between adsorption affinity and degree of randomness in cellulose hydrolysis, measured as the diversity of released hydrolytic products. Kinetic measurements indicated that only part of the adsorbed enzyme molecules are hydrolytically active.

INTRODUCTION

Cellulose is a renewable carbohydrate source and available in large quantities. Many attempts have been made to utilize this enormous amount of carbohydrate through enzymatic hydrolysis into glucose. Cellulases, capable of hydrolysis of crystalline cellulose are derived from several fungi (1-5).The extracellular multi-enzyme system of these micro-organisms is characterized by three main components: the 1,4-β-D-glucan glucanohydrolase or endoglucanase (EC 3.2.1.4), the 1,4-β-D-glucan cellobiohydrolase or exoglucanase (EC 3.2.1.91) and the β-D-glucoside glucohydrolase or β-glucosidase (EC 3.2.1.21). The multiplicity of the enzyme complex and the synergistic action between endoglucanase and exoglucanase (6) cause many difficulties in

studying the mode of action of the complex.

The substrate-enzyme system is heterogeneous and consists of the water insoluble cellulose and the water soluble enzymes. This implies that the first step in cellulolysis is binding of the cellulase enzymes on the surface of the cellulose fibrils. After binding of the enzyme molecules the actual catalytic action, i.e. the hydrolysis of the susceptible glucosidic bonds, takes place. The physicochemical heterogeneity of the cellulose introduces another multiplicity, which has large influence on the adsorption characteristics of the cellulase enzymes, as well as on the extent of hydrolysis.

Crystallinity of cellulose was shown to be the main parameter which determines its biodegradability (7, 8). Besides of the enzyme and cellulose concentration, the amount of adsorbed cellulase per unit mass of cellulose, depends on the crystallinity index and the specific surface area. The adsorption equilibrium constant, obtained from the Langmuir-type adsoption isotherm, using a constant cellulose concentration, decreases with decreasing crystallinity index and the maximum protein adsorption constant increases with increasing specific surface area (9, 10). At present all the research carried out on adsorption of cellulase on cellulosic substrates has been done with the complete culture filtrate (9-14), commercial enzyme preparations (15) or partially purified and characterized endoglucanase and exoglucanase fractions (16). Information on the adsorption of various endoglucanase and exoglucanase components of these preparations was obtained from activity measurements on carboxymethylcellulose (endoglucanase) and filterpaper (exoglucanase) (11-14). However, it has been proven that such assays are unsuitable for calculations of enzyme quantities (17, 18).

The work presented in this paper avoids such drawbacks by the use of highly purified enzymes. The adsorption characteristics and the reaction kinetic parameters of six endoglucanases and two exoglucanases, purified from a commercial cellulase preparation, derived from Trichoderma viride (19) were determined on crystalline Avicel cellulose. The adsorption behaviour of endoglucanases were compared to their mode of action in cellulose hydrolysis, especially with respect to the release of soluble products.

MATERIALS AND METHODS

Enzymes

Six endoglucanases (Endo I, II, III, IV, V and VI) and two exoglucanases (Exo II and III) were isolated from the commercial cellulase preparation Maxazyme CL (Gist-Brocades, Delft, the Netherlands) as described before (19). If necessary the enzyme solutions were concentrated to the desired protein concentration (1-2 mg/ml) by ultrafiltration (Diaflo Type 50, Amicon, Mass., USA) using the PM10 membrane.

Adsorption studies

Various amounts of the purified endoglucanases, or exoglucanases and appropriate amounts of 0.05 M sodium acetate buffer pH 5.0 were mixed with a fixed amount of Avicel cellulose (Type SF, Serva, Heidelberg, F.R.G.) to give a final volume of 0.5 ml. The enzyme protein concentration varied between 0.1 and 1.0 mg/ml. The final cellulose concentration was 20 mg/ml. Incubation took place for 1 hour at 30 ^{o}C in 0.05M sodium acetate pH 5.0 with continuous mixing on a roller drum (New Brunswick Scientific, Edison, USA). After centrifugation of the cellulose (10 min, 3000 x g), the supernatant was analysed on non-adsorbed protein using the Folin reagent (20). Cellulase, adsorbed on cellulose was estimated by substracting the amount of dissolved protein from the values obtained in a similar experiment without cellulose.

Reversibility of the adsorption was studied under conditions of about 50 % saturation. The cellulose was centrifuged off, followed by replacement of two-third of the total volume by fresh buffer. After resuspending the cellulose, further incubation took place during 1 and 8 hours, using the same conditions. The samples were centrifuged again and the free protein concentration was determined.

Kinetic studies

The reaction mixtures for kinetic studies on Avicel cellulose consisted of various concentrations of substrate (11.43 to 90.91 mg/ml) and a fixed enzyme concentration (25µg/ml) in 0.05M sodium acetate buffer pH 5.0. The final volume was 1 ml. Incubation took place on a roller drum at 30^{o}C for 20 hours. After centrifugation aliquots of the reaction mixtures were taken and analysed for reducing sugars by the method of Nelson and Somogyi (21).

Hydrolysis products analysis

The six endoglucanases were incubated with 2% Avicel cellulose in a final volume of 0.5 ml at 30°C in 0.05 M sodium acetate buffer for 6 hours. The enzyme concentration was 60 µg/ml. After centrifugation of the cellulose, aliquots of 0.4 ml were taken from the supernatant, the enzymes were inactivated by placing the tubes in a boiling water-bath for 5 min. and evaporated with dry air. The residue was dissolved in 75 µl 0.1 M $Pb(NO_3)_2$ and centrifuged to remove solids. Products in the supernatant were analysed by HPLC (Spectra Physics SP 8000) using the Aminex HPX 87P column (300 x 7.8 mm, Bio-Rad Labs, Richmond CA, USA) coupled with a guard column, containing equal amounts of the ion-exchange resins AG 50W-X4 (H^+) and AG3-X4A (OH^-) (Bio Rad labs). The column was operated with water as eluent at 85°C and a flow rate of 0.5 ml/min. Detection took place with an ERMA-ERL 7510 refractive index detector, thermostated at 40°C.

RESULTS

Adsorption studies

After incubation of various amounts of purified endoglucanase or exoglucanase with a fixed amount of Avicel cellulose the free protein concentration P (mg/ml) was determined. The quantity of adsorbed enzyme was calculated and P_{ads} was defined as the amount of adsorbed protein divided through the amount of cellulose (mg protein/mg cellulose). From these data the adsorption isotherms (P_{ads} vs P) were constructed for the endoglucanases (Fig. 1) and exoglucanases (Fig. 2).

On the basis of these isotherms two groups of endoglucanases could be distinguished. One group, consisting of Endo I, Endo III and Endo V adsorbed strongly on crystalline cellulose, while another group of endoglucanases, Endo II, Endo IV and Endo VI, adsorbed moderately (Endo II) or only very slightly. From the two exoglucanases Exo III appeared to adsorb much stronger than Exo II.

Adsorption data of the complete cellulase complex are known to obey the Langmuir-type adsorption isotherm (10, 15, 22, 24):

$$P_{ads} = \frac{K_p \cdot P_{ads,m}}{1 + K_p \cdot P} P \qquad [1]$$

where P is the free protein concentration (mg/ml); P_{ads} is the amount of adsorbed protein (mg adsorbed protein/mg cellulose). $P_{ads,m}$ is the maximal amount of adsorbed protein (mg adsorbed protein/mg cellulose) and Kp is the adsorption equilibrium constant $(mg/ml)^{-1}$.

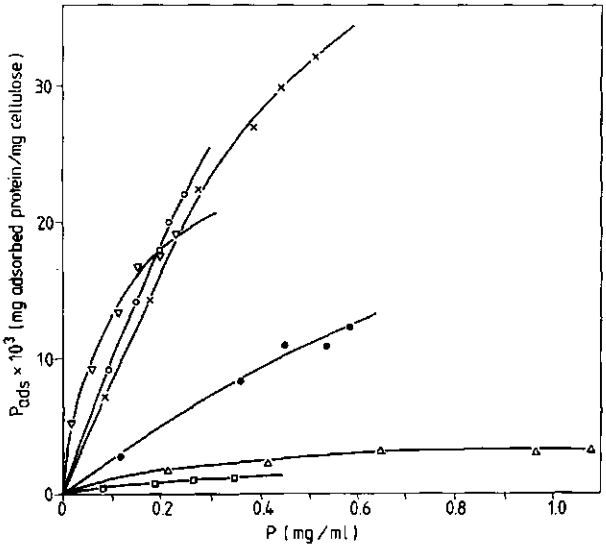
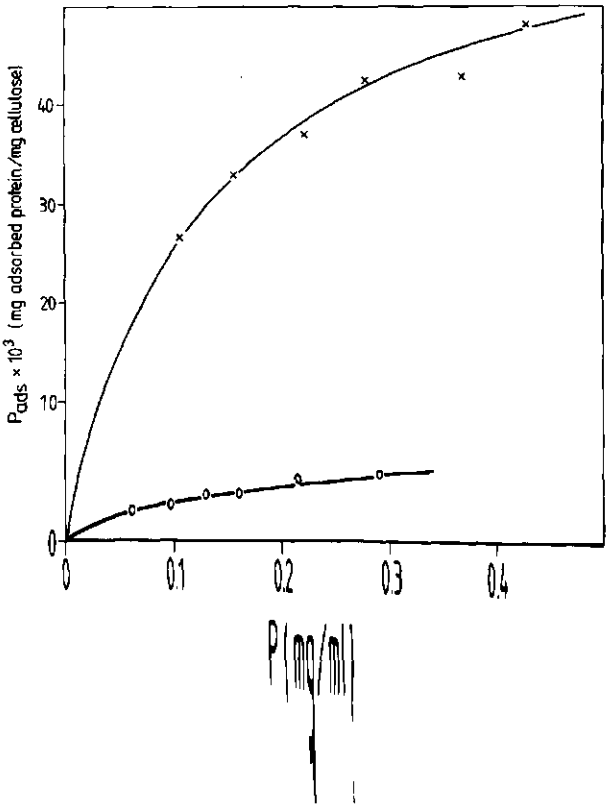


Fig. 1. Adsorption isotherms of purified endoglucanases on Avicel crystalline cellulose (20 mg/ml). o, Endo I; ●, Endo II; ▽, Endo III; □, Endo IV; ×, Endo V; △, Endo VI.



Hydrolysis products analysis

The six endoglucanases were incubated with 2% Avicel cellulose in a final volume of 0.5 ml at 30°C in 0.05 M sodium acetate buffer for 6 hours. The enzyme concentration was 60 µg/ml. After centrifugation of the cellulose, aliquots of 0.4 ml were taken from the supernatant, the enzymes were inactivated by placing the tubes in a boiling water-bath for 5 min. and evaporated with dry air. The residue was dissolved in 75 µl 0.1 M $Pb(NO_3)_2$ and centrifuged to remove solids. Products in the supernatant were analysed by HPLC (Spectra Physics SP 8000) using the Aminex HPX 87P column (300 x 7.8 mm, Bio-Rad Labs, Richmond CA, USA) coupled with a guard column, containing equal amounts of the ion-exchange resins AG 50W-X4 (H^+) and AG3-X4A (OH^-) (Bio Rad labs). The column was operated with water as eluent at 85°C and a flow rate of 0.5 ml/min. Detection took place with an ERMA-ERL 7510 refractive index detector, thermostated at 40°C.

RESULTS

Adsorption studies

After incubation of various amounts of purified endoglucanase or exoglucanase with a fixed amount of Avicel cellulose the free protein concentration P (mg/ml) was determined. The quantity of adsorbed enzyme was calculated and P_{ads} was defined as the amount of adsorbed protein divided through the amount of cellulose (mg protein/mg cellulose). From these data the adsorption isotherms (P_{ads} vs P) were constructed for the endoglucanases (Fig. 1) and exoglucanases (Fig. 2).

On the basis of these isotherms two groups of endoglucanases could be distinguished. One group, consisting of Endo I, Endo III and Endo V adsorbed strongly on crystalline cellulose, while another group of endoglucanases, Endo II, Endo IV and Endo VI, adsorbed moderately (Endo II) or only very slightly. From the two exoglucanases Exo III appeared to adsorb much stronger than Exo II.

Adsorption data of the complete cellulase complex are known to obey the Langmuir-type adsorption isotherm (10, 15, 22, 24):

$$P_{ads} = \frac{K_p \cdot P_{ads,m}}{1 + K_p \cdot P} P \qquad [1]$$

where P is the free protein concentration (mg/ml); P_{ads} is the amount of adsorbed protein (mg adsorbed protein/mg cellulose). $P_{ads,m}$ is the maximal amount of adsorbed protein (mg adsorbed protein/mg cellulose) and Kp is the adsorption equilibrium constant $(mg/ml)^{-1}$.

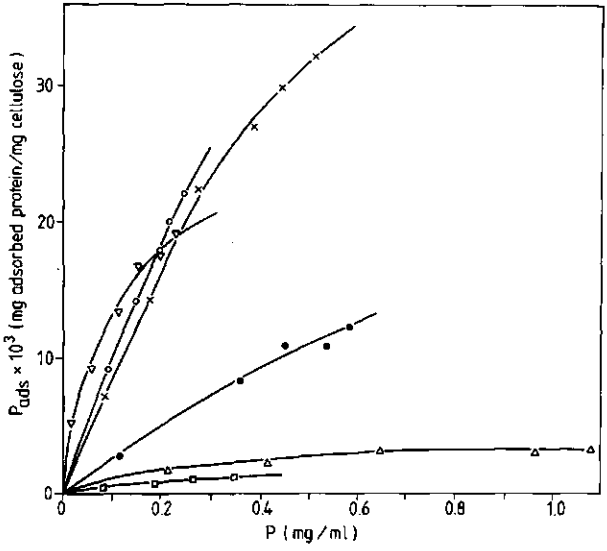


Fig. 1. Adsorption isotherms of purified endoglucanases on Avicel crystalline cellulose (20 mg/ml). ○, Endo I; ●, Endo II; ▽, Endo III; □, Endo IV; ×, Endo V; △, Endo VI.

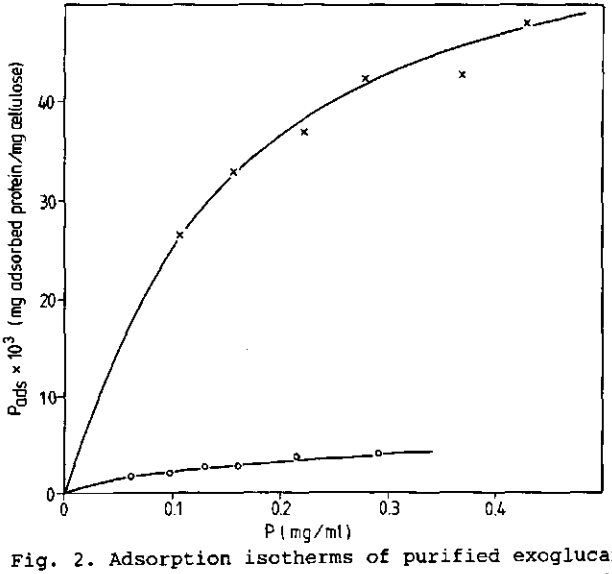


Fig. 2. Adsorption isotherms of purified exoglucanases on Avicel crystalline cellulose (20 mg/ml). ○, Exo II; ×, Exo III.

Rearrangement of eq.[1] gives:

$$\frac{P}{P_{ads}} = \frac{1}{K_p \cdot P_{ads,m}} + \frac{1}{P_{ads,m}} P \quad [2]$$

A plot of P/P_{ads} versus P will give a straight line, from which the adsorption parameters $P_{ads,m}$ and K_p can be calculated. Using eq. [2] for the experimental data of adsorption of the purified endoglucanases and exoglucanases, straight lines were obtained (Fig. 3, 4, 5), indicating that these data fit the Langmuir-type adsorption isotherm indeed. Adsorption parametrers, obtained from these lines are presented in Table I.

Reversibility of adsorption is one of the conditions which have to be obeyed to attach any physical significance to the adsorption parameters obtained from the Langmuir eq. [1]. Therefore reversibility was investigated by the centrifugation of cellulose and replacement of a large part of the supernatant, containing non-adsorbed protein, by fresh buffer. After

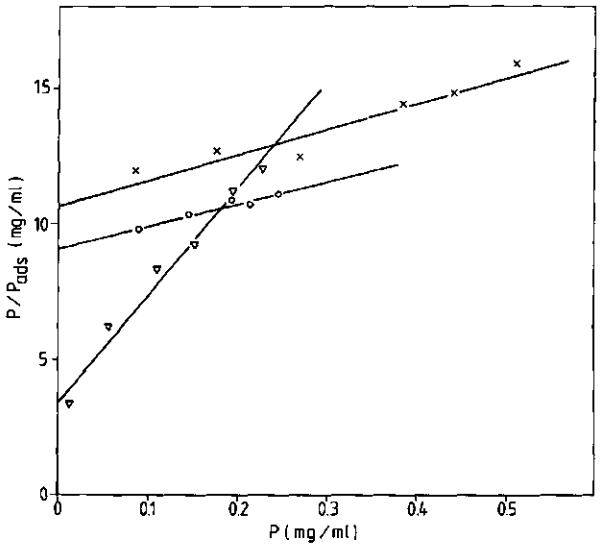


Fig. 3. Langmuir-type adsorption plots of Endo I (O), Endo III (∇) and Endo V (x).

resuspending the cellulose and further incubation, the amount of released protein (ΔP_{ads}) was measured and expressed as percentage of the amount of protein, which theoretically could desorb at full reversibility, using the Langmuir-type isotherm (Table II).

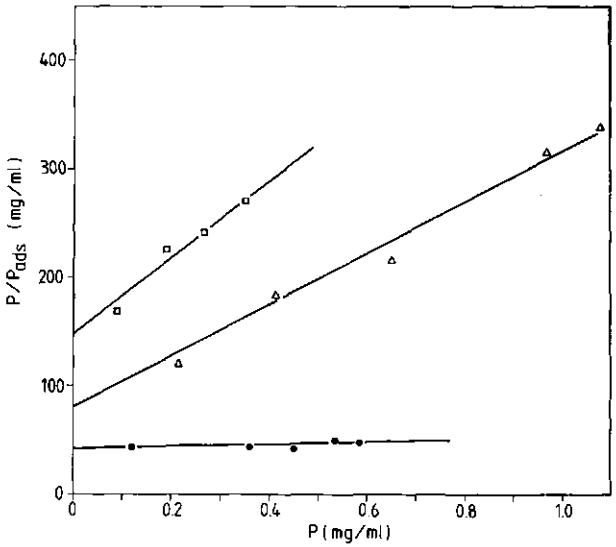


Fig. 4. Langmuir-type adsorption plots of Endo II (●), Endo IV (□) and Endo VI (△).

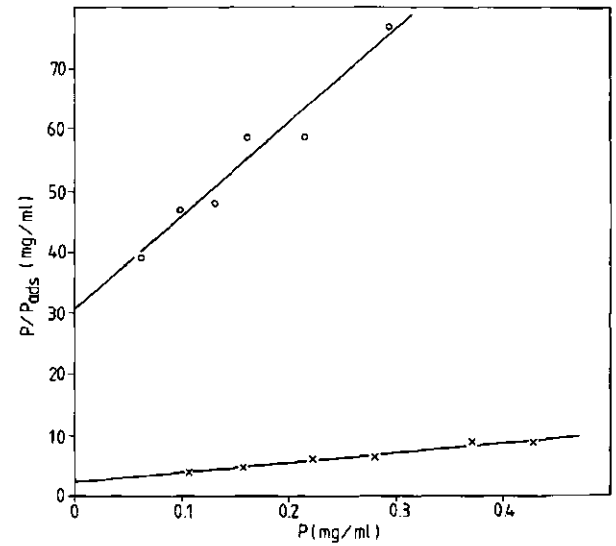


Fig. 5. Langmuir-type adsorption plots of Exo II (O) and Exo III (X).

Table I. Adsorption parameters and reaction kinetic parameters of purified endo- and exoglucanases from Trichoderma viride.

Enzyme	K_p $(mg/ml)^{-1}$	$P_{ads,m}$ (mg.protein/ mg cellulose)	R $(mg/ml)^{-1}$	K_m (mg/ml)	V_{max} (µmol/ mg.min)
Endo I	0.88	0.126	0.111	46.3	0.196
Endo II	0.28	0.090	0.025	90.6	0.099
Endo III	11.67	0.026	0.303	14.4	0.085
Endo IV	2.50	0.0028	0.007	130.7	0.152
Endo V	0.89	0.105	0.094	64.3	0.133
Endo VI	3.44	0.0041	0.014	122.9	0.122
Exo II	4.96	0.0066	0.037	44.1	0.060
Exo III	6.92	0.063	0.436	12.0	0.019

Table II. Degree of reversibility of adsorption. Amount of desorbed enzyme protein (ΔP_{ads}) at 1 hour desorption and calculated values at full reversible reaction; n.d., not determined.

Enzyme	ΔP_{ads}		% reversibility
	measured	calculated from Langmuir isotherm	
Endo I	1.10	4.10	27
Endo II	2.19	5.06	43
Endo III	1.82	3.15	58
Endo IV	n.d.	n.d.	n.d.
Endo V	0.91	1.99	46
Endo VI	n.d.	n.d.	n.d.
Exo II	n.d.	n.d.	n.d.
Exo III	10.39	13.81	75

Adsorption of Endo I, II, III, V and Exo III was only partially reversible. Even a longer desorption time up to 8 hours did not increase the amount of released protein (data not shown). Exo III gave the highest level of reversibility: 75% of the amount of protein, which could theoretically desorb at full reversible reaction, was released. Data on desorption of Endo IV, VI and Exo II are not given because these enzymes adsorb only slightly. Therefore detection of the small amounts of these enzymes which could desorb was not possible, using the methods described.

Because adsorption of at least some of the endoglucanases and exoglucanases is not completely reversible, no physical significance can be attributed to the Langmuir constants, calculated from eq.[2] and presented in Table I. Therefore Kp can not interpreted as an affinity constant of adsorption. As a parameter which describes the degree of interaction between the enzymes and the cellulose, R was introduced and defined as the slope of the isotherm at its origin (P=0), where no interaction between the enzyme molecules takes place. This slope equals the inverse intercept of the lines obtained from eq.[2] (Fig. 3, 4, 5), so:

$$R = K_p \cdot P_{ads,m} \qquad [3]$$

R has the dimension of (mg cellulose/ ml)$^{-1}$. From Table 1 it can be seen that the strongest interaction between the endoglucanases and cellulose was found for Endo III. Also Endo I and Endo V showed a relatively large R-value. These latter enzymes have, in comparison with Endo III, a larger $P_{ads,m}$, which means that more adsorption sites on the cellulose chains are available. However, in the case of Endo I and Endo V, the affinity of enzyme to substrate is low and high initial concentrations of these enzymes are needed to reach saturation.

Small values for R were obtained for Endo II, IV and VI. Although interaction between Endo II and Avicel is rather poor, relatively many adsoption sites for this enzyme are present on this type of cellulose (high $P_{ads,m}$). For Endo IV and VI, however, the amount of cellulose which was used in these experiments was already saturated by these enzymes at a low protein concentration. Exo III is the most abundant exoglucanase in the commercial cellulase preparation from which it was purified (19) and showed an interaction with Avicel which was much stronger than for Exo II. Also the saturation level of adsorption for Exo III was about ten times higher than the value found for Exo II.

Kinetic studies

Hydrolysis kinetics by the endo- and exoglucanases was studied using Avicel cellulose as the substrate. The kinetic parameters K_m and V_{max} were obtained from the Lineweaver-Burk plots of the experimental data (Fig. 6, 7) and presented in Table I. For the different endoglucanases the lowest K_m values were found for those enzymes which have a high initial rate of adsorption, i.e. Endo I, III and V (Table I). The same observa-

tion was made for the two exoglucanases. The lower K_m of Exo III is attended with the higher value for R.

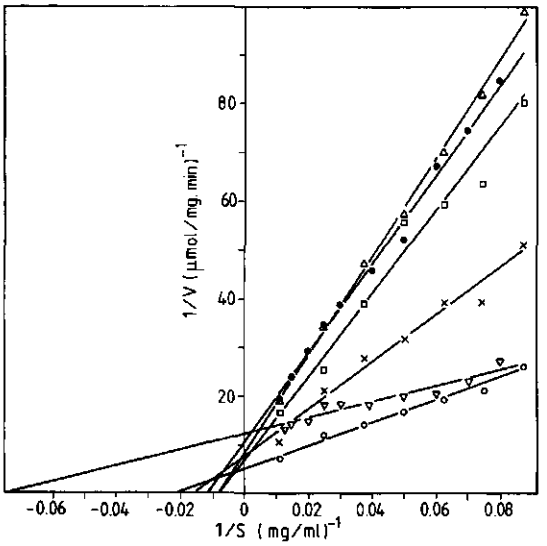


Fig. 6. Lineweaver-Burk plots of purified endoglucanases using Avicel crystalline cellulose as the substrate. o, Endo I; ●, Endo II; ▽, Endo III; □, Endo IV; ×, Endo V; △, Endo VI.

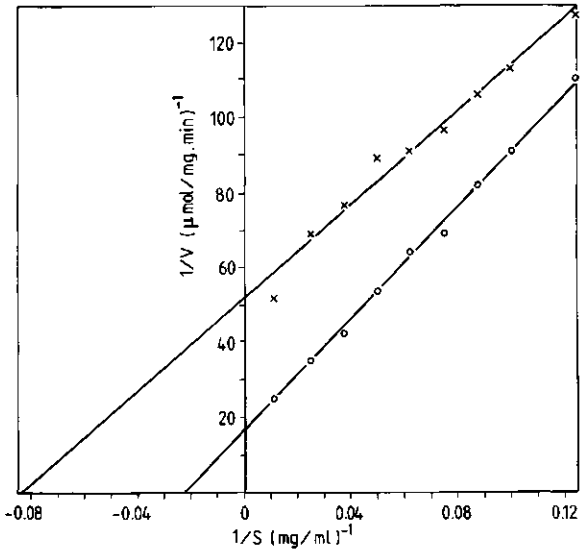


Fig. 7. Lineweaver-Burk plots of purified exoglucanases. o, Exo II; ×, Exo III.

Hydrolysis products analysis

Hydrolysis products from incubations of the endoglucanases with Avicel cellulose were analysed on HPLC and presented in Figure 8. Endo I, II, III and IV produced a mixture of glucose, cellobiose and cellotriose. Small amounts of cellotetraose were found in the reaction products of Endo IV after a very short incubation time (1 hour; data not shown). Only glucose and cellobiose were obtained from incubations with Endo V and Endo VI. The presence of transglucosidase activity could be excluded, because relative peak areas after 2 and 6 hours of incubation were comparable (data not shown).

The differences which were observed in adsorption behaviours of the endoglucanases (Fig. 1) did not parallel the diversity in hydrolytic action of these enzymes. From the endoglucanases I, III and V which do adsorb to a great extent, Endo I and III produce G_2, G_3 and G_4, while Endo V produces only G_2 and G_1. On the other hand, from the endoglucanases which both produce only G_2 and G_1 during cellulose hydrolysis, one (Endo V) adsorbs strongly on its substrate, while the other enzyme (Endo VI) does not.

The different degrees of random attack during cellulose hydrolysis of these endoglucanases results in reaction mixtures

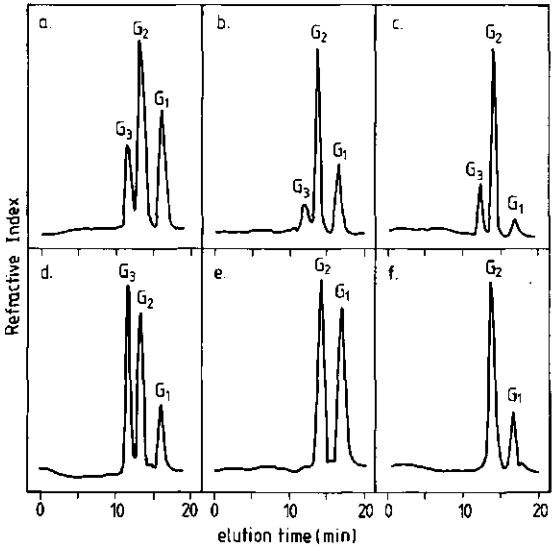


Fig. 8. HPLC analysis of products released from Avicel crystalline cellulose by Endo I (a), Endo II (b), Endo III (c), Endo IV (d), Endo V (e) and Endo VI (f). G_1, glucose; G_2, cellobiose; G_3, cellotriose.

with a more or less diverse composition (G_4, G_3, G_2 and G_1) or in a more homogeneous reaction product containing only G_2 and G_1. However, it appears that the degree of randomness is not determined by the adsorption characteristics.

DISCUSSION

Several investigators showed that adsorption of cellulase on crystalline cellulose is a relatively fast process. Ooshima et al. (15) stated that the equilibrium of adsorption of Trichoderma viride cellulase on pure Avicel cellulose was reached within 30 minutes at temperatures between 5-50°C. A contact time of 15 minutes was found to be sufficient to attain a steady state of adsorption in a mixture of Talaromyces emersonii cellulase and a number of cellulosic substrates with different compositions (12). For the experiments described here, a contact time of 1 hour was used to establish equilibrium.

The results clearly show that the group of purified endoglucanases, as well as the two exoglucanases, can be divided into two subclasses of enzymes: those enzymes which do adsorb strongly (Endo I, III, V, and Exo III) and those which absorb only slightly or moderately on crystaline cellulose (Endo II, IV, VI and Exo II). The existence of a non-adsorbing endoglucanase component in a cellulase complex of T. reesei was also observed by Ryu et al. (16). The results in this paper indicate that such a non-adsorbing endoglucanase component consists primarily of distinct, specific endoglucanases.

One of the consequences of such a specific adsorption for the recovery of the complete cellulase complex after (incomplete) cellulose hydrolysis, is that the enzyme composition of the adsorbed fraction can be completely different from the composition of the original complex. This should be considered when recovery of enzymes is done by adsorption on fresh substrate as suggested by some authors (11).

Also the reversibility of the adsorption was examined. It appeared that at least a part of the enzyme molecules was irreversibly adsorbed on the cellulose chains, even after prolonged desorption time. However, the experimental data of the adsorption experiments, fortuitously fitted the Langmuir-type adsorption isotherm (eq.[1]), which makes this equation useful for calculations of the amounts of adsorbed cellulase protein,

at the cellulose concentration (20 mg/ml) used in these experiments.

This finding was used to evalutate the reaction kinetic experiments. It can be assumed that at a very high cellulose concentration ($S \rightarrow \infty$) all of the available enzyme (P_{av}) is adsorbed to the substrate and the maximal reaction rate (V_{max}) is achieved. It is reasonable to suppose that, going from low to high substrate concentration an increase of reaction rate parallels an increase of the amount of adsorbed enzyme. Lee et al. (10) showed that this was true for adsorption of the complete cellulase complex of T. reesei. Also assuming that the distribution of the active and possible inactive enzymes remains unchanged during adsoption, one can expect that, at a certain cellulose concentration, the part of total available enzyme which is adsorbed, expressed as P_{ads}/P_{av}, equals the ratio of actual reaction rate and maximal reaction rate (i.e. V/V_{max}) at that specific cellulose concentration. This latter ratio can be obtained directly from the kinetic experiments, while eqs.[1] makes it possible to calculate the ratio of adsorbed and available enzyme protein at a substrate concentration of 20 mg/ml.

It can be concluded from Table III that for most of the purified endoglucanases and exoglucanases these ratios are not equivalent. Especially for those enzymes which do adsorb to a great extent i.e. Endo I, III, IV and Exo III, it can be estimated that a large part of the available enzyme is already adsorbed at this cellulose concentration, while the reaction rate for these enzymes under these conditions was only a much smaller part of their V_{max}. For instance from Endo I 69% of the available enzyme protein was adsorbed, but only 31% of the V_{max} was achieved.

Table III. Part of total avaiblable enzyme which is adsorbed and part of maximal reaction rate at a cellulose concentration of 20 mg/ml.

Enzyme	P_{ads}/P_{av}	V/V_{max}
Endo I	0.69	0.31
Endo II	0.33	0.18
Endo III	0.85	0.58
Endo IV	0.12	0.13
Endo V	0.65	0.24
Endo VI	0.21	0.14
Exo II	0.38	0.31
Exo III	0.90	0.63

An explanation for this discrepancy may be that a part of the adsorbed enzyme molecules are not bound at their catalytic site. An other explanation is that these enzymes do indeed adsorb at their catalytic site, but are not able to express their hydrolytic action. It may be possible that the activity of these enzymes - for instance an endoglucanase - will only find expression if an other enzyme, in this case an exoglucanase, is also present. On the other hand part of the exoglucanase activity will only be measured if an endoglucanase molecule adsorbs at the same spot and creates a new chain-end. This phenomenon may explain partially the synergism between endo- and exoglucanases.

In a previous study (Chapter 4) we showed that different degrees of randomness in the attack of cellulose chains by endoglucanases exist (19). This is expressed as the ratio of the liquefying activity to the saccharifying activity. The value of this ratio was shown to be the highest for Endo I, Endo II and Endo IV. The results in this paper show that these cellulases indeed are the most random types of the six endoglucanases which were purified from Trichoderma viride, because a considerable amount of cellotriose was produces by these enzymes. Low liquefying/saccharifying values were obtained for Endo V and Endo VI, which is consistent to the fact that these enzymes gave only cellobiose and glucose as hydrolysis product. One could expect that an enzyme which is tightly adsorbed at its solid substrate is less mobile and therefore less random in hydrolytic action than an enzyme which adsorbs only slightly. However, no such relationship could be observed for the endoglucanases investigated in this study. Randomness is rather determined by the fact how the enzyme structurally binds to its substrate, than by how strongly it binds. The extremely low degree of randomness in cellulose attack by Endo III (19) is in contrast with the heterogeneous composition of its hydrolysis product (G_3, G_2 and G_1). Possibly this can be explained by a single chain multiple attack mechanism of this enzyme, contrary to the multiple chain attack of the other endoglucanases.

NOMENCLATURE

P	free enzyme (mg/ml)
P_{ads}	adsorbed enzyme (mg/mg cellulose)
P_{av}	available enzyme (mg/mg cellulose)
$P_{ads,m}$	maximum adsorbed enzyme (mg/mg cellulose)
K_p	adsorption equilibrium constant $(mg/ml)^{-1}$
R	initial rate of adsorption (mg cellulose/ $ml)^{-1}$
V	actual reaction rate (μmol/mg.min)
V_{max}	maximal reaction rate (μmol/mg.min)
Km	Michaelis-Menten constant (mg/ml)

REFERENCES

1. Mandels, M., Meideiros, J.E., Andereotti, R.E. and Bisset, F.H., Biotechnol. Bioeng. 1981, 23, 2009-2026
2. Tangu, S.K., Blanch, H.W. and Wilke, C.R. Biotechnol. Bioeng. 1981, 23, 1837-1849
3. Shin, S.B., Kitakawa, Y., Suga, K.I. and Ichikawa, K., J. Ferment. Technol. 1978, 56, 396-402
4. Wood, T.M., Biochem. J. 1968, 109, 217-227
5. Eriksson, K.E. and Pettersson, B., Eur. J. Biochem. 1975, 51, 193-206
6. Wood, T.M. and McCrae, S.I., Biochem. J. 1978, 171, 61-72
7. Fan, L.T., Lee, Y.H. and Beardmore, D.H., Biotechn. Bioeng. 1980, 22, 177-199
8. Ohmine, K., Ooshima, H. and Harano, Y., Biotechn. Bioeng. 1983, 25, 2041-2053
9. Lee, Y.H. and Fan, L.T., paper presented at the Sixth International Fermentation Symposium, London, Ontario, Canada, 1980
10. Lee, S.B., Shin, H.S. and Ryu, D.D.Y. Biotechnol. Bioeng. 1982, 24, 2137-2153
11. Castanon, M., and Wilke, C.R. Biotechnol. Bioeng. 1980, 22, 1037-1053
12. Moloney, A. and Coughlan, M.P. Biotechnol. Bioeng. 1983, 25, 271-280
13. Goel, S.C. and Ramachandran, K.B. J. Ferment. Technol. 1983, 61, 281-286
14. Deshpande, V., Mishra, C., Ghadge, G.D., Secta, R. and Rao, M., Biotechnol. Lett. 1983, 5, 391-394
15. Ooshima, H., Sakata, M and Harano, Y., Biotechnol. Bioeng. 1983, 25, 3103-3114
16. Ryu, D.D.Y., Kim, C. and Mandels, M., Biotechnol. Bioeng. 1984, 26, 488-496
17. Lindner, W.A., Dennison, C. and Quicke, G.V. Biotechnol. Bioeng. 1983, 25, 377-385
18. Canevascini, G. and Gattlen, C. Biotechnol. Bioeng. 1981, 23, 1573-1590
19. Beldman, G., Searle-van Leeuwen, M.F., Rombouts, F.M. and Voragen, A.G.J., Eur. J. Biochem. 1985, 146, 301-308

20. Lowry, O.H. Rosebrough, N.J., Farr, A.L. and Randell, R.J., J. Biol. Chem. 1951, 193, 262-275
21. Somogyi, M., J. Biol. Chem. 1952, 195, 19-23
22. Peitersen, N., Medeiros, J. and Mandels, M., Biotechnol. Bioeng. 1977, 19, 1091-1094
23. Reese, E.T., Process Biochemistry May/June, 2-6 (1982).
24. Stuart, J.Y. and Ristroph, D.L., Biotechnol. Bioeng. 1985, 27, 1056-1059

6 Synergism in cellulose hydrolysis by endoglucanases and exoglucanases purified from *Trichoderma viride*

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SYNERGISM IN CELLULOSE HYDROLYSIS BY ENDOGLUCANASES AND EXO-GLUCANASES PURIFIED FROM TRICHODERMA VIRIDE

G. Beldman, A.G.J. Voragen, F.M. Rombouts and W.Pilnik.

Department of Food Science, Agricultural University, Wageningen.

Synergistic effects in cellulose hydrolysis between six endoglucanases (Endo I, II, III, IV, V and VI) and two exoglucanases (cellobiohydrolases Exo II and Exo III), which were purified from a commercial cellulase preparation, derived from Trichoderma viride were investigated. Especially Exo III acted strongly synergistically with all endoglucanases. The degree of synergistic effect was largely dependent upon the initial ratio of endoglucanase to exoglucanase and maximal at a specific value, which could qualitatively be related to the adsorption behaviours of the endoglucanases. Those endoglucanases which adsorb strongly on crystalline cellulose (Endo I, III and VI) needed to be used in smaller amounts to reach maximal degree of synergism, than Endo II, IV and VI which we have found are to adsorb less. These results indicate that only those endoglucanase and exoglucanase molecules which are adsorbed on the cellulose chains as a complex with a specific ratio of both enzymes are involved in synergistic effects on insoluble crystalline cellulose.
Probably because of cellobiase activity, another exoglucanase (Exo I) showed synergistic action when working in concert with Exo II and Exo III.

INTRODUCTION

One of the most intriguing phenomena in cellulose hydrolysis by the cellulases of several fungi is the synergistic action between the individual components of these enzyme mixtures. The enzymes which play a main role in synergism are the endoglucanase [1,4-β-D-glucan glucanohydrolase (EC 3.2.1.4)] and the exoglucanase [1,4-β-D-glucan cellobiohydrolase (EC 3.2.1 .91.)]. Both types of enzymes were found to exist in multiple forms in a.o. the culture filtrates of Trichoderma viride (1-3), Trichoderma Koningii (4, 5), Sporotrichum pulverulentum (6),

Penicillium funiculosum (7, 8), Fusarium solani (9) and Talaromyces emersonii (10).

Synergism in cellulose hydrolysis by the cellulase complex of several of these fungi has been demonstrated by recombination experiments of the purified endo- and exoglucanases (4, 5). Also "cross-synergism" has been observed between the endo- and exo-acting enzymes from different microbial origins (7). Synergism in the enzymatic degradation of crystalline cellulose is not simply explained by the sequential attack of endoglucanases and exoglucanases. For instance it was found by Wood et al.(5) that one of the four endoglucanases from T. Koningii, which was the most random acting enzyme in cellulose attack, and therefore expected to create relatively many chain-ends for an exo-glucanase to act on, showed only very little synergistic effect when working together with the cellobiohydrolase from this fungus. These authors state that the formation of a complex of endoglucanase and cellobiohydrolase on the surface of the cellulase chain is essential for synergism.

From the mechanistic model described by Okazaki et al. (11) it was concluded that the degree of synergistic effect (DSE), which is defined as the ratio of the observed activity of the combined endo- and exoglucanase enzymes, to the sum of the observed individual activities, largely depends on the ratio of endoglucanase and exoglucanase, as well as on the degree of polymerization of the substrate. The model was used to predict synergistic effects, using kinetic data obtained from incubations with soluble substrates. The value of this model to predict a degree of synergistic effect (DSE) during hydrolysis of insoluble crystalline cellulose is questionable.

An explanation for synergism from another point of view was given by Ryu et al. (12), who studied the sequential adsorption of the endoglucanase and exoglucanase components of T. reesei and found that synergism was gradually "introduced" into the non-adsorbed enzyme fraction by competitive adsorption of one of the glucanases. These investigators state that synergism of the cellulase complex remains undisturbed during adsorption, but show no data on synergism between cellulases in the adsorbed fraction. The work was done with partially purified enzymes, which still contained several iso-enzymes. In two previous papers we described the purification and biochemical characterization of all endoglucanases and exoglucanases of a commercial enzyme preparation from T. viride (1), as well as the adsorption and kinetic behaviours of these enzymes (13). This paper

presents the results of the recombination experiments with these purified enzymes. The synergistic effects which were observed could partially be explained in the light of the reported adsorption behaviours of these cellulases.

MATERIALS AND METHODS

Enzyme preparations

Maxazyme Cl is a commercial cellulase preparation from Trichoderma viride origin and was provided by Gist-Brocades, Delft, the Netherlands. From this preparation six endoglucanases (Endo I, II III, IV, V and VI) and three exoglucanases (Exo I, Exo II and III) were isolated as described in a previous paper (1).

Recombination experiments

Endoglucanases in combination with Exo III on Avicel cellulose. From the endoglucanase preparations (Endo I, II, III, IV, V, VI) a fixed amount of 90 µg enzyme was combined with an amount of Exo III ranging from 6 to 200µg protein. Incubation with 0.5% Avicel cellulose (Type SF, Serva, Heidelberg, F.R.G.) took place under continuously mixing on a roller drum during 20 hours at 30°C in 0.05 M sodium acetate buffer. The final volume was 1 ml. In a parallel experiment the endoglucanases and Exo III were incubated separately using the same conditions as in the combination experiment. After incubation the cellulose was centrifuged off (10 min., 3000 x g) and the supernatant was analysed on released reducing sugars by the method of Nelson Somogyi (14) as well as on total sugars by the method of Dubois et al. (15).

Endo IV in combination with Exo III on H_3PO_4-swollen cellulose. Recombination of Endo IV with Exo III was carried out as described above, using 0.5% H_3PO_4-swollen cellulose instead of Avicel. The H_3PO_4-swollen cellulose was prepared by the method of Wood (16).

Fixed amounts of endoglucanases or Exo I in combination with Exo II and Exo III on Avicel cellulose. Endoglucanases or Exo I (25 µg) were combined with 25 µg of respectively Exo II or Exo III. Incubation of the combinations as well as of the separate enzymes took place during 20 hours at 30°C in a volume of 1 ml 0.05M sodium acetate pH 5.0 with 0.5% Avicel cellulose. After removal of residual cellulose, the amount of

reducing sugars in the supernatant was determined.

Endo III in combination with Endo I on Avicel cellulose. Endo III was recombined with Endo I, using 90 µg of both enzymes, and incubated at 30°C with 0.5% Avicel during 20 hours. The final volume was 1 ml 0.05M sodium acetate pH 5.0. The same incubation was carried out with the separate enzymes. Products were analysed as described above.

Endoglucanases or Exo III in combination with Maxazyme Cl on Avicel cellulose. Maxazyme Cl, in a final concentration of 500 µg/ml, was combined with the individual endoglucanases, respectively Exo III, and incubated at 30°C in 0.05M sodium acetate buffer pH 5.0. The purified glucanases were used in a concentration of 77 µg/ml. After incubation during 20 hours the cellulose was centrifuged and the amount of reducing sugars in the supernatant was determined and compared to the values obtained from separate incubations of Maxazyme Cl and purified cellulases, using the same conditions.

RESULTS

Endoglucanases in combination with Exo III

Recombination of the purified exoglucanase Exo III and endoglucanases Endo I, II, III, IV, V and VI resulted in all cases in a synergistic effect after incubation with Avicel crystalline cellulose. The degree of synergistic effect (DSE) could be calculated by comparing the activity of the combinations to the sum of the activities of the individual enzymes. By using a fixed concentration of the endoglucanases and a varying concentration of Exo III it was found that the DSE depends upon the endoglucanase to exoglucanase ratio.

Figure 1 shows that for all combinations of endoglucanase with Exo III a maximum DSE was observed at a specific ratio of these enzyms. The maximal value of DSE was dependent upon the methods of analysis of the hydrolysis products. Analysis of released reducing sugars, which gives a value for every glucosidic bond which is enzymatically cleaved, resulted in maximal DSE values varying between 1.60 (Fig. 1C) and 3.45 (Fig. 1E).

Determination of total sugars by the method of Dubois represents a solubilization effect of the enzymatic degradation of cellulose and gives, with the exception of the combination of

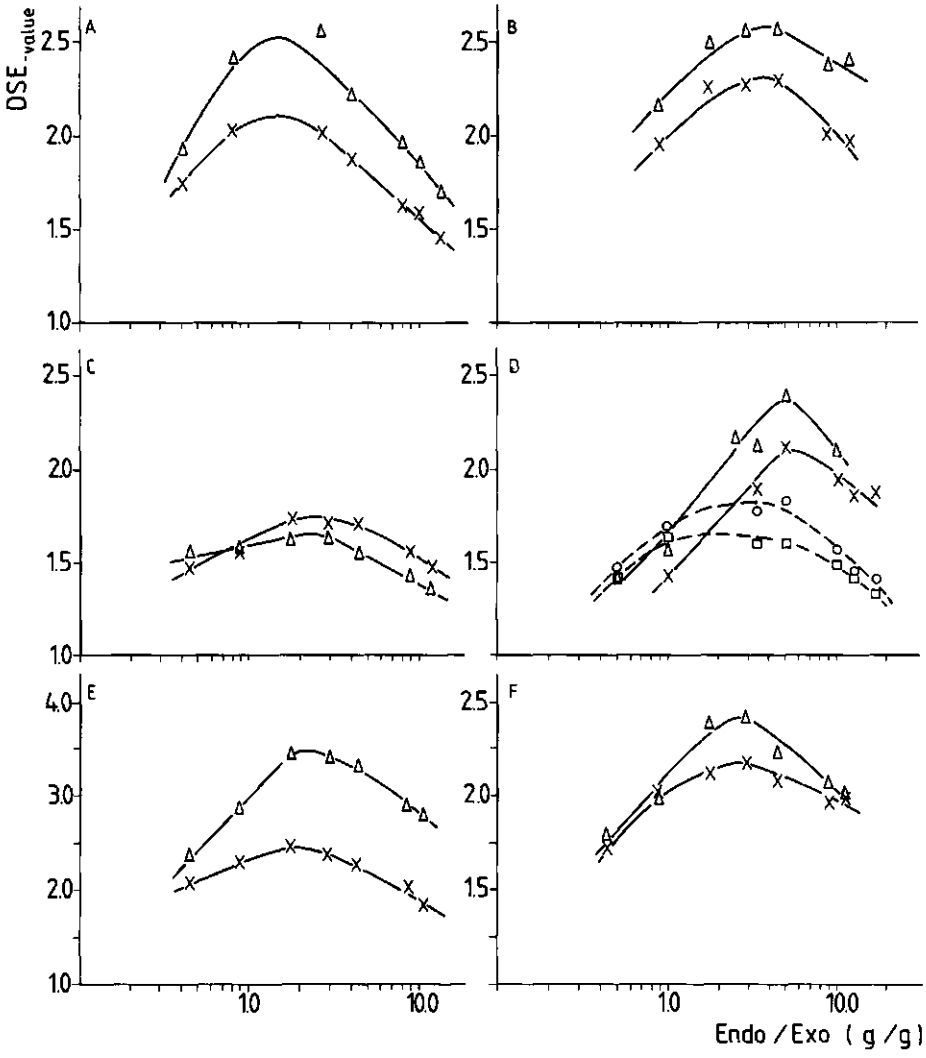

Fig. 1. Degree of synergistic effect (DSE), obtained after recombination of Exo III with various amounts of Endo I (A), Endo II (B), Endo III (C), Endo IV (D), Endo V (E) and Endo VI (F), during hydrolysis of Avicel (A-F) and H_3PO_4-swollen cellulose (D). Products were measured as reducing sugars (RS) as well as total sugars (TS). Δ, Avicel, RS; ×, Avicel, TS; ○, H_3PO_4-swollen cellulose, RS; □, H_3PO_4-swollen cellulose, TS

Endo III with Exo III (Fig. 1C) lower maximal DSE values between 1.75 and 2.45. The weight-ratio of endoglucanase to exoglucanase where maximal DSE occurs, is given in Table I. Taken into account the molecular masses of these enzymes (1), also the molar ratios could be calculated. In our previous study on adsorption of purified endoglucanases and exoglucanases, we showed that Endo I, III and V adsorb strongly on Avicel cellulose. Especially these enzymes show their maximal DSE at a much lower endoglucanase to exoglucanase ratio than Endo II, IV and VI when working in concert with Exo III.

Synergistic effects in hydrolysis of amorphous H_3PO_4-swollen cellulose was investigated for the combination of Endo IV and Exo III. From Figure 1D it can be seen that the combination of these enzymes results in DSE values, which are much lower than those values obtained in hydrolysis of Avicel cellulose. Also a much broader maximum was observed, at a lower

Table I. Initial endoglucanase to Exo III ratios, at which maximal DSE was observed under the experimental conditions.

Exo III in combination with	Endo/Exo III	
	mass ratio	molar ratio
Endo I*	1.5	1.9
Endo II	3.75	5.2
Endo III*	2.50	2.7
Endo IV	5.0	13.2
Endo V*	2.0	2.2
Endo VI	2.75	3.3

* Adsorb strongly on Avicel cellulose (13).

Table II. DSE-values obtained for the combination of endoglucanases and Exo I with Exo II and Exo III using an enzyme mass ratio of 1.

Enzyme	Combination with	
	Exo II	Exo III
Endo I	0.9	1.5
Endo II	1.4	2.1
Endo III	1.0	1.2
Endo IV	1.2	1.7
Endo V	1.3	2.0
Endo VI	1.3	1.6
Exo I	1.4	3.5

endoglucanase to exoglucanase ratio.

Endoglucanases and Exo I in combination with Exo II and Exo III. The two cellobiohydrolases Exo II and Exo III, which were isolated from Maxazyme Cl, were compared with respect to their capacities to act synergistically when working together with the endoglucanases and Exo I on Avicel cellulose (Table II). DSE-values measured for the combinations of Exo II with these enzymes were found to be lower than the values obtained in a similar experiment using Exo III. Actually no synergism was found for the combination of Exo II with Endo I and Endo III. The enzyme concentration used in these experiments was 25 µg/ml for each enzyme (Endo/Exo = 1). At this protein concentration a combination of an endoglucanase with Exo III exhibited a lower DSE-value than observed in the previous experiment in which a protein concentration of 90 µg/ml was used for both enzymes. Exo I, which we found to be an enzyme able to release cellobiose from cellulose and subsequently hydrolyse this product to glucose, worked strongly synergistically with Exo III.

Endo III in combination with Endo I. In a previous paper (1; Chapter 4) some doubts remained on the endo/exoglucanase nature of Endo III. In order to investigate whether or not Endo III is a true endoglucanase, the ability of this enzyme to act synergistically with Endo I was studied. Endo I was shown to be a true endoglucanase (1). A DSE-value of 0.97 was found, indicating that these two enzymes are not able to act synergistically and Endo III cannot be classified as an exoglucanase.

Endoglucanases and Exo III in combination with Maxazyme Cl. The synergistic effects which could be obtained with a combina-

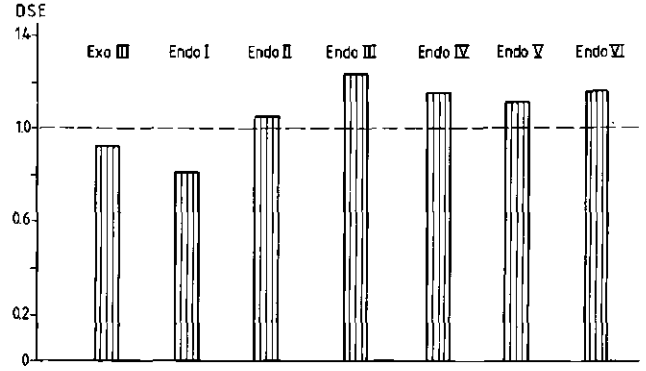

Fig. 2. Degree of synergistic effect (DSE), obtained after recombination of the original cellulase preparation Maxazyme Cl with purified endoglucanases and Exo III.

tion of two purified cellulases, was also studied in much more complex mixture of these types of enzymes. Maxazyme Cl, the original cellulase preparation from which the endo- and exo-glucanases were purified, was supplemented with the individual enzymes and investigated on its Avicel hydrolysing capacity, in comparison with the activities obtained in separate incubations. Figure 2 shows that endoglucanases II, III, IV, V and VI are able to increase the activity of the original cellulase preparation, by acting synergistically with it. The DSE-values obtained varied between 1.06 for a combination with Endo II to 1.24 when Endo III was combined with Maxazyme CL. Endo I and Exo III, however, gave a negative synergistic effect when supplemented to the original cellulase preparation.

DISCUSSION

It is shown that all six endoglucanases (Endo I, II, III, IV, V, VI) which were isolated from the commercial cellulase preparation Maxazyme Cl, can act synergistically in cellulose hydrolysis with the cellobiohydrolase Exo III, obtained from the same preparation. The degree of synergistic effect (DSE) for a specific combination of endo- and exoglucanase, is at least dependent upon two main factors: firstly the ratio in which both enzymes are combined and secondly the nature of the substrate which is used (in these experiments crystalline Avicel or amorphous H_3PO_4-swollen cellulose).

The fact that the DSE obtains a maximal value at one specific endoglucanase to exoglucanase ratio is in good agreement with the theoretical model of Okazaki (11), who stated that enzymatic cellulolysis is strongly affected by this ratio, as well as by the degree of polymerization of the substrate. However, these authors used their model on the basis of soluble substrates, assuming homogeneous reaction conditions. When insoluble crystalline cellulose is used, the reaction is heterogeneous and another factor is introduced: the selective adsorption characteristics of the individual endo- and exoglucanases.

The results, presented in this paper, show that for hydrolysis of Avicel cellulose the optimal initial endoglucanase to exoglucanase ratio is greatly dependent upon the adsorption behaviours of these enzymes. Endo I, Endo III and Endo V, which enzymes are known to adsorb strongly on Avicel (13), exhibited

their maximal synergistic effect with Exo III (which also adsorbs strongly on Avicel) at a much lower initial endo- to exoglucanase ratio than in those incubations where Endo II, Endo IV and Endo VI were used. It was shown in our previous work that these latter endoglucanases adsorb only slightly on Avicel. The observations can be explained by assuming that only those cellulases which are adsorbed on the insoluble cellulose chains, participate in the formation of a complex of endo- and exo-glucanase, which is responsible for the synergistic effect. In agreement to the findings of Wood et al. (4), the activity of an exoglucanase in this complex can only find expression after the formation of a new chain-end by the endoglucanase. On the other hand the action of an endoglucanase can only be expressed if it is immediately followed by the removal of a cellobiose molecule, as the result of the action of an exoglucanase, in order to prevent the reformation of the first broken bond.

Endoglucanases such as Endo II, Endo IV and Endo VI need to be added in a relatively high concentration to create a "driving force" which is strong enough for the adsorpion of at least a part of the available enzyme molecules and the formation of the endoglucanase-cellobiase complex. The results indicate that maximal DSE occurs at a specific optimal ratio of adsorbed endo-glucanases and exoglucanases. It is very likely that the endo- and exoglucanase need to be present in such a complex in a 1 : 1 ratio, in order to obtain a maximal DSE.

It is clear that this optimum depends more upon the adsorp-tion characteristics, than upon the hydrolytic nature (specific activity, degree of randomness, oligosaccharide composition of the product) of the individual enzymes. These latter properties of the enzymes determine a.o. the different DSE-values at optimal ratio of the various combinations as well as the differences between DSE-values obtained by measurement of the product as reducing sugars (bond-breaking activity) or total sugars (solubilization activity) for one specific combination. The fact that Endo III, in combination with Exo III gives a relatively low DSE-value at the optimal ratio, may be related to the low degree of randomness in cellulose attack of this enzyme (1). We suggested that this enzyme acts according to a single chain mutiple attack mechanism (13), which is in accordance to the fact that the DSE-value, measured as bond-breaking activity and as solubilization activity, differ only slightly for the combination of this enzyme with Exo III. This may be explained by the fact that Endo III is more or less restricted to the same

cellulose chain in a crystalline matrix and solubilization parallels hydrolytic activity. In contrast, the action of the other endoglucanases (Endo I, II, IV, V, VI) in contrary, which jump from chain to chain (multiple-chain attack), will not always lead to solubilization, because the chain is fixed in a crystalline matrix. However, it can result in a new chain-end for an exoglucanase to act on, creating new measurable reducing end-groups.

The fact that synergism between Endo IV and Exo III on amorphous H_3PO_4-swollen cellulose is less striking than on Avicel is in good agreement with data from the literature (12) . We also found that DSE on this type of amorphous cellulose was to a lesser extent determined by the ratio of both enzymes, which is expressed in a broader maximum (Fig. 1D). Probably more encounters of one of these enzymes and this type of more or less soluble substrate lead to the release of a measurable product. This effect is only slightly increased by the addition of the complementary enzyme. This type of synergism takes place more often in solution (soluble synergism) while synergism on Avicel especially takes place on the crystalline surface (insoluble synergism).

The endoglucanase nature of Endo III was discussed in our previous study (1) since this enzyme exhibited a relatively low activity towards CM-cellulose and a high activity towards Avicel, compared with the other endoglucanases. By the observation that synergism was positive with Exo III and negative in combination with Endo I, we confirm its endoglucanase nature.

The synergistical action of Exo I with the cellobiohydrolyses Exo II and Exo III can at least partially be explained by the fact that cellobiose produced by these latter enzymes is hydrolysed by Exo I to two glucose units giving more reducing sugars and eliminating possible product inhibition. However, it may be possible that synergism between these exoglucanases is similar to the synergistic action of two cellobiohydrolases described by Fägerstam and Pettersson (17), although these authors also found glucose in the enzyme digest, suggesting similarities to our observations.

Synergism can also be observed after addition of purified cellulases to a more complex mixture of these enzymes. Supplementing Maxazyme Cl with the endoglucanases II, III, IV, V and VI resulted in a higher activity than the sum of activities obtained in the separate incubations. One can conclude that Maxazyme Cl is deficient in these endoglucanases. However, this

effect cannot be generalized since addition of Endo I gave a negative synergistic effect, indicating again that synergism is more than simple successive action of endoglucanase and exoglucanase, but rather related to other properties, such as adsorption nature, of these enzymes. During hydrolysis of cellulose by complex mixtures of cellulases, such as culture filtrates and commercial enzyme preparations, there will be a competition between the individual enzymes to occupy the available adsorption sites and to form the endoglucanase/ exoglucanase complex, mentioned above. Taking into account that, in the case of T. viride, at least eight enzymes with different adsoption parameters are involved, one can conclude that it is very difficult to predict the optimal composition of an enzyme mixture in order to obtain maximal synergism.

REFERENCES

1. Beldman, G., Searle-van Leeuwen, M.F., Rombouts, F.M. and Voragen, A.G.J., Eur. J. Biochem. 1985, 146, 301-308
2. Schoemaker, S.P. and Brown, R.D., Biochem. Biophys. Acta 1978, 523, 133-146
3. Schoemaker, S.P. and Brown, R.D., Biochem. Biophys. Acta 1978, 523, 147-161
4. Wood, T.M. and McCrae, S.I., Proc. Bioconvers. Symp. I.I.T., New Delhi, 1977, pp 111-141.
5. Wood, T.M. and McCrae, S.I., Biochem. J. 1978, 171, 61-72
6. Eriksson, K.E. and Pettersson, B., Eur. J. Biochem. 1975, 51, 193-206
7. Wood, T.M., McCrae, S.I. and Macfarlane, C.C., Biochem. J. 1980, 189, 51-65
8. Wood, T.M. and McCrae, S.I., Carbohydr. Res. 1982, 110, 291-303
9. Wood, T.M. and McCrae, S.I., Carbohydr. Res. 1977, 57, 117-133
10. McHale, A. and Coughlan, M.P., FEBS-Lett. 1980, 117, 319-322
11. Okazaki, M. and Moo-Young, M., Biotechn. Bioeng. 1978, 20, 637-663
12. Ryu, D.D.Y., Kim, C. and Mandels, M., Biotechn. Bioeng. 1984, 26, 488-496
13. Beldman, G., Voragen, A.G.J., Rombouts, F.M., Searle-van Leeuwen, M.F. and Pilnik, W., accepted for publication in Biotechn. Bioeng. 1986
14. Somogyi, M., J. Biol. Chem. 1952, 195, 19-23
15. Dubois, M., Gilles, K.A., Hamiltion, J.K., Rebers, F.A. and Smith, F., Anal. Chem. 1956, 28, 350-356
16. Wood, T.M., Biochem. J. 1971, 121, 353-362
17. Fägerstam, L.G. and Pettersson, L.G., FEBS Lett. 1980, 153, 113-118

7 Specific and non-specific glucanases from *Trichoderma viride*

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SPECIFIC AND NON-SPECIFIC GLUCANASES FROM TRICHODERMA VIRIDE

G. Beldman, A.G.J. Voragen, F.M. Rombouts, M.F. Searle-van Leeuwen and W. Pilnik.

Department of Food Science, Agricultural University, Wageningen

Six endoglucanases (Endo I, II, III, IV, V and VI), three exo-glucanases (Exo I, II and III) and a β-glucosidase (β-gluc I) which were isolated from a commercial cellulase preparation of Trichoderma viride origin, were examined as to their activities on xylan ex oat spelts. Endo I, II and III, as well as Exo II and III showed no activity towards xylan and were classified as specific glucanases. Less specificity was found for the endo-glucanases Endo IV, V and VI, Exo I and β-gluc I which enzymes were able to hydrolyse xylan. With respect to product formation these xylanolytic cellulases fit the classification of xylanases generally accepted in the literature. Kinetic experiment with xylan, CM-cellulose and p-nitrophenyl-β-D-glucoside revealed that Endo IV, V an VI and Exo I prefer to hydrolyse β-1,4-D-glucosidic linkages. β-gluc I showed no clear substrate preference.

INTRODUCTION

Many micro-organisms are able to produce a wide variety of polysaccharide degrading enzymes. Important groups of these types of enzymes are cellulases and xylanases, because they can potentially be employed to utilize two of the most abundant polysaccharides, respectively cellulose (1-3) and xylan (4-6). Hydrolases from fungal origin have been studied extensively. Many authors reported on the purification and characterization of cellulases (7-14) and xylanases (4, 5, 15-19) from these sources. Substrate cross-specificity of cellulases and xylanases is often observed. For instance small amounts of cellulase activity were found in homogeneous xylanases, purified from crude enzyme mixtures obtained from Aspergillus niger (4, 18, 19), Trichoderma viride (15) and Oxiporus sp. (15). On the other hand homogeneous cellulases from commercial enzyme preparations derived from Irpex lacteus (9) or Trichoderma viride (8)

exhibited some xylanase activity. Isoelectric focussing of a mixture of endo-1,4-β-glucanases and endo-1,4-β-xylanases from Trichoderma reesei showed the possibility to distinguish between specific xylanases, specific glucanases and non-specific glucanases, applying a zymogram method with dyed substrates (5).

There is some debate on the nature of the non-specific glucanases found in the culture filtrates of Trichoderma. Sprey and Lambert (20) were able to split a cellulase complex, which was homogeneous on isoelectric focussing, into several proteins with different isoelectric points and distinct activities on CM-cellulose, xylan and 4-methylumbelliferyl-β-D-glucoside. A mixture of 6M urea and 1% octylglucoside was needed to split the complex. These results indicate that the fungus releases a tightly bound complex of several hydrolases in order to attack a multi-component substrate of plant cell-wall polysaccharides. Shikata and Nisizawa (8) also found a cellulase which showed xylanase and β-glucosidase activity. However, these investigators concluded from a competition experiment, using CM-cellulose, xylan and p-nitrophenyl-β-D-glucoside, that these three substrates react at the same active site of the enzyme.

This conclusion is supported by the fact that both substrates have an analogous structure. The main chain of xylan consists of β-D-xylopyranosyl units, which are 1,4-β-linked, comparable to the β-1,4-linked glucose residues of cellulose. However, xylan is a more heterogeneous polysaccharide and usually branched (5). Dependent on the source from which it is extracted, xylan containes branches with various amounts of L-arabinofuranosyl and D-xylo-, D-gluco-, and D-galactopyranosyl units. Also D-glucuronic and 4-O-methyl-D-glucuronic acid residues are linked to the main chain. Moreover, xylans from several kinds of wood are esterified with acetic acid.

In previous reports we described the purification and characterization of six endoglucanases, three exoglucanases and a β-glucosidase from Trichoderma viride (7). Also the adsorption and reaction kinetics (21), as well as the synergistic behaviours (22) of these enzymes were described. The aim of the present work was to investigate the specific or non-specific nature of these glucanases, by determination of their activities towards xylan and several xylo- and arabino-xylo-oligosaccharides.

MATERIALS AND METHODS

Enzyme preparations

Six endoglucanases (Endo I, II, III, IV, V and VI), three exoglucanases (Exo I, II and III) and a β-glucosidase (β-gluc I) were purified from Maxazyme CL (Gist-Brocades, Delft, the Netherlands), a commercial cellulase preparation from Trichoderma viride origin, as previously reported (7).

Substrates

CM-cellulose, type Akucel AF 0305 was from Akzo, Arnhem, the Netherlands. Xylan ex oat spelts was from Koch-Light, Colnbrook, Bucks, England. This material was characterized by methylation analysis as described by Talmadge et al. (23) and had a composition as presented in Table I. Almost 90% of the sugar residues are β-D-(1,4)-linked xylose units, of which about 13% have single or double branch points.

Enzyme incubations with xylan

The endoglucanases, exoglucanases and β-glucosidase were incubated at 30°C in 0.1 M sodium acetate buffer pH 5.0 with 1 mg/ml xylan. The enzyme concentration was 60 µg/ml in a final volume of 1 ml. After an incubation time of 3 and 21 hours the reaction was stopped by addition of 0.1 ml 1.0 M lead nitrate. The precipitate which was formed at 4-6°C was centrifuged. The samples which were incubated during 3 hours were concentrated

Table I. Types of linkages present in xylan ex oat spelts (mole percentages).

Sugar residue	Types of linkages	mole %
D-Xylose	O-1	4.0
	O-1, O-4	75.7
	O-1, O-3, O-4	9.9
	O-1, O-2, O-4	1.5
	O-1, O-2, O-3, O-4	0.6
L-Arabinose	O-1	5.1
	O-1, O-2	1.8
	O-1, O-3	0.2
	O-1, O-5	0.5
D-Glucose	O-1, O-4	0.6
D-Galactose	O-1, O-3, O-6	0.1

three times by evaporation and centrifuged again to remove additional precipitate. The clear supernatant was analysed by HPLC, using a Spectra Physics model SP 8000, equiped with an Aminex HPX 87P column (300 x 7.8 mm, Bio-Rad Labs, Richmond LA, USA) as described earlier (21; Chapter 5).

Preparation of oligomers from xylan

Xylo- and arabinoxylo-oligosaccharides were prepared on a larger scale for the use of peak identification in the HPLC analysis and as a substrate for further enzyme incubation. Cellulases, exhibiting xylanase activity, were incubated with xylan in a volume of 10 ml, using the conditions as mentioned above. After 21 hours of incubation the enzymes were inactivated by placing the tubes in a boiling water bath for 5 min. Insoluble residues were removed by centrifugation and the supernatant was concentrated ten times by evaporation under vacuum. The concentrate was fractionated on a Bio-Gel P2 (200-400 mesh, Bio-Rad Labs) column (100 x 1.0 cm) using degassed distilled water as eluent. Fractions of 0.52 ml were collected and analysed for sugars by the phenol-sulphuric acid assay (24). Peaks were analysed for their sugar compositions by gaschromatography after hydrolysis in 2 N tri-fluor acetic acid and derivatization to alditol acetates (25). The chromatografic behaviours of the mono- and oligosaccharide fractions obtained from the gel permeation chromatography were also determined by HPLC.

Enzyme incubations with oligosaccharides

Purified oligosaccharides obtained from the degradation product of xylan with Endo IV were re-incubated with those cellulases, which were found to be active on xylan. Incubation took place with an oligosaccharide concentration of 1.3 mg/ml and an enzyme concentration of 30 µg/ml, during 20 hours at 30°C in 0.1 M sodium acetate buffer pH 5.0. Inactivation, sample preparation and HPLC analysis was carried out as described above.

Kinetic experiments

Reaction kinetic parameters of the purified cellulases were determined on CM-cellulose for Endo I, II, III, IV, V and VI and on xylan and p-nitrophenyl-β-D-glucoside (pNp-Gluc) for those enzymes, which were shown to be active on these types of substrates. The incubation with CM-cellulose took place at 30°C

in 0.05 M sodium acetate buffer pH 5.0 using several substrate concentrations between 1.3 and 8.0 mg/ml and an enzyme concentration of 1 µg/ml. The reaction mixtures for kinetic studies on xylan consisted of various concentrations of substrate, ranging from 1.9 to 15.4 mg/ml and an enzyme concentration of 2 µg/ml in 0.1 M sodium acetate pH 5.0. The incubation took place at 30°C. After the desired reaction times, aliquots of the reaction mixtures were taken and analysed for reducing sugars by the method of Nelson and Somogyi (26). The samples were centrifuged before reading absorbance at 520 nm. Incubation with pNp-Gluc was done under the same conditions with a substrate concentration between 0.03 and 30 mg/ml. The concentration of p-nitrophenol was measured after 1 hour at 400 nm, using the absorption coefficient of 13700 $M^{-1}cm^{-1}$.

RESULTS

Enzyme incubations with xylan

From the ten glucanases, purified by us from a commercial cellulase preparation derived from Trichoderma viride (7) only five enzymes showed activity towards xylan (Table II). Even after prolonged incubation (21 hours) with Endo I, Endo II, Endo III, Exo II and Exo III no degradation products from xylan could be observed by HPLC. From Figure 1 it can be seen that Endo IV released mainly xylobiose (X_2) and xylotriose (X_3) from xylan. The ratio of these oligosaccharides remained about equal after a longer incubation period.

The identification of the oligomer peaks was done with standards, obtained from gel permeation chromatography on Bio-Gel P2 (see next paragraph). More diversity of hydrolysis products was observed after incubation of xylan with Endo V. Although X_2 was the main product, also xylose (X_1), X_3 and xylotetraose (X_4) were detected. The larger oligomer was not found after 21 hours of incubation. On short term Endo VI produced a mixture of X_1, X_2, X_3 and X_4, however this enzyme was also able to degrade these oligomers to monomeric xylose, which was found as the only product after prolonged incubation. Since xylan ex oat spelts contains also a small amount of β-D-(1,4)-linked glucose (Table I), which can be cleaved off by these enzymes, some glucose (G_1) was found in almost all hydrolysates. Besides G_1 and X_n an amount of arabinose (A_1) was released

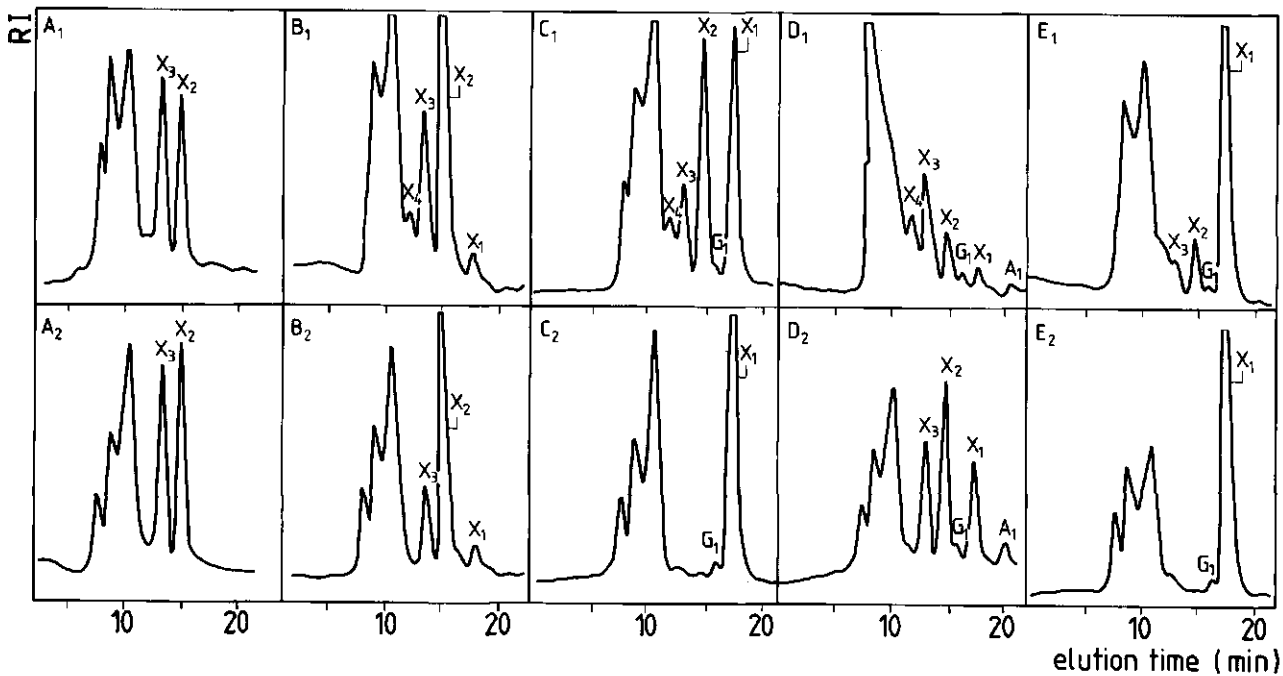

Fig. 1. HPLC analysis of products released from xylan by Endo IV (A_1; A_2), Endo V (B_1; B_2), Endo VI (C_1; C_2), Exo I (D_1; D_2) and β-gluc I (E_1; E_2). Incubation took place during 3 hours (A_1-C_1) and 21 hours (A_2-C_2).

Table II. Activity of cellulases from Trichoderma viride on xylan. P, positive; N, negative.

Enzyme	Activity
Endo I	N
Endo II	N
Endo III	N
Endo IV	P
Endo V	P
Endo VI	P
Exo I	P
Exo II	N
Exo III	N
β-gluc I	P

from the polymeric substrate by Exo I. With the exception of a small amount of G_1, X_1 and X_2, β-gluc I produced xylose as the only hydrolysis product.

Preparation of oligomers from xylan

Gel permeation chromatography on Bio-Gel P2 was used to prepare xylo- and arabinoxylo-oligomers from xylan. These oligomers were produced for two reasons: firstly as standards for peak identification in HPLC analysis and secondly as substrates for further enzymatic conversion. Figure 2 shows the chromatograms of the digests of xylans with those cellulases which were shown to be active on this substrate, i.e. Endo IV, Endo V, Endo VI, Exo I and β-gluc I. The elution profiles are in good agreement with the results presented in Figure 1, however, since Bio-Gel P2 separates these saccharides only on the basis of molecular mass differences and because xylan is a heterogeneous substrate, the peaks are not necessarily homogeneous.

Therefore samples of the peaks were further characterized with respect to the sugar compositions as well as by HPLC analysis of the oligomers. The results are summarized in Table III and some typical chromatograms are given in Figure 3. The dimer and trimer fractions obtained from the digestion of xylan with Endo IV, Endo V, Endo VI and Exo I, consisted of pure xylose and gave single peaks by HPLC analysis at retention times of respectively 15.05 (Figs. $3A_2$ and $3B_2$) and 13.35 min. (Figs. $3A_3$ and $3B_3$). Therefore these peaks were identified as X_2 and X_3. The pentamer peaks from the Bio-Gel P2 chromatography of xylan digests with Endo IV, Endo V and Endo VI consisted of xylose and arabinose in a ratio of 4 : 1 and gave single peaks at a retention time of 10.73 min., when subjected to HPLC

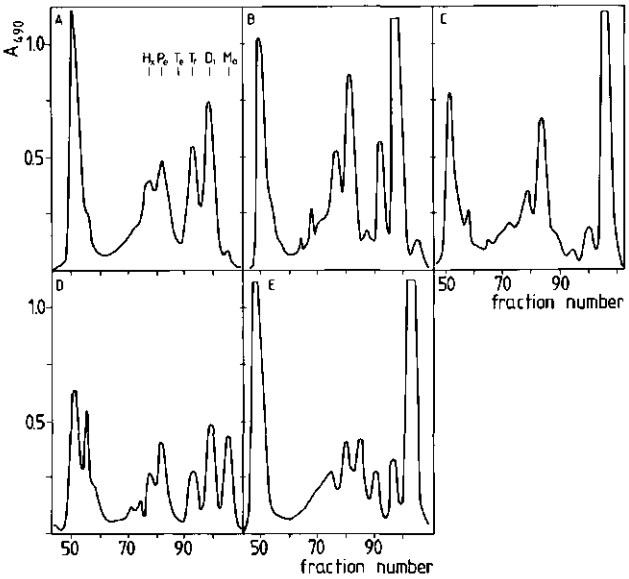

Fig. 2. Bio-Gel P2 chromatography of products released from xylan by Endo IV (A), Endo V (B), Endo VI (C), Exo I (D) and β-gluc I (E). Mo, monomer; Di, dimer; Tr, trimer; Te, tetramer; Pe, pentamer; Hx, hexamer.

Table III. Sugar composition and identification of oligosaccharides from P2-gelpermeation chromatochraphy, obtained after incubation of xylan with several cellulases, purified from Trichoderma viride. SC, Sugar composition of the peaks from P2-column; X, 100% xylose; X:A, ratio of xylose to arabinose residues; RT, Retention time on HPLC analysis; OC, Oligosaccharide composition of the peaks from P2-column.

Enzyme	Dimer	Trimer	Tetramer	Pentamer	Hexamer
Endo IV	SC X	X		X:A=4:1	X:A=5:1
	RT 15.05	13.35		10.62	10.15
	OC X_2	X_3		X_4A_1	X_5A_1
Endo V	SC X	X		X:A=4:1	X:A=5:1
	RT 15.05	13.35		10.73	10.15
	OC X_2	X_3		X_4A_1	X_5A_1
Endo VI	SC X			X:A=4:1	X:A=5:1
	RT 15.05			10.73	10.20
	OC X_2			X_4A_1	X_5A_1
Exo I	SC X	X		X:A=3:1	X:A=4:1
	RT 14.98	13.42		10.73	10.26
	OC X_2	X_3		X_4A_1 + X_3A_2	X_5A_1 + X_4A_2
β-gluc I	SC X	X:A=6:1	X:A=6:1	X:A=5:1	X:A=5:1
	RT 10.26 14.98	12.07 13.35	12.07 11.32	10.90	10.08
	OC X_2 X_2	X_2A_3 X_3	X_4 X_3A_1	X_4A_1 + X_5	X_5A_1

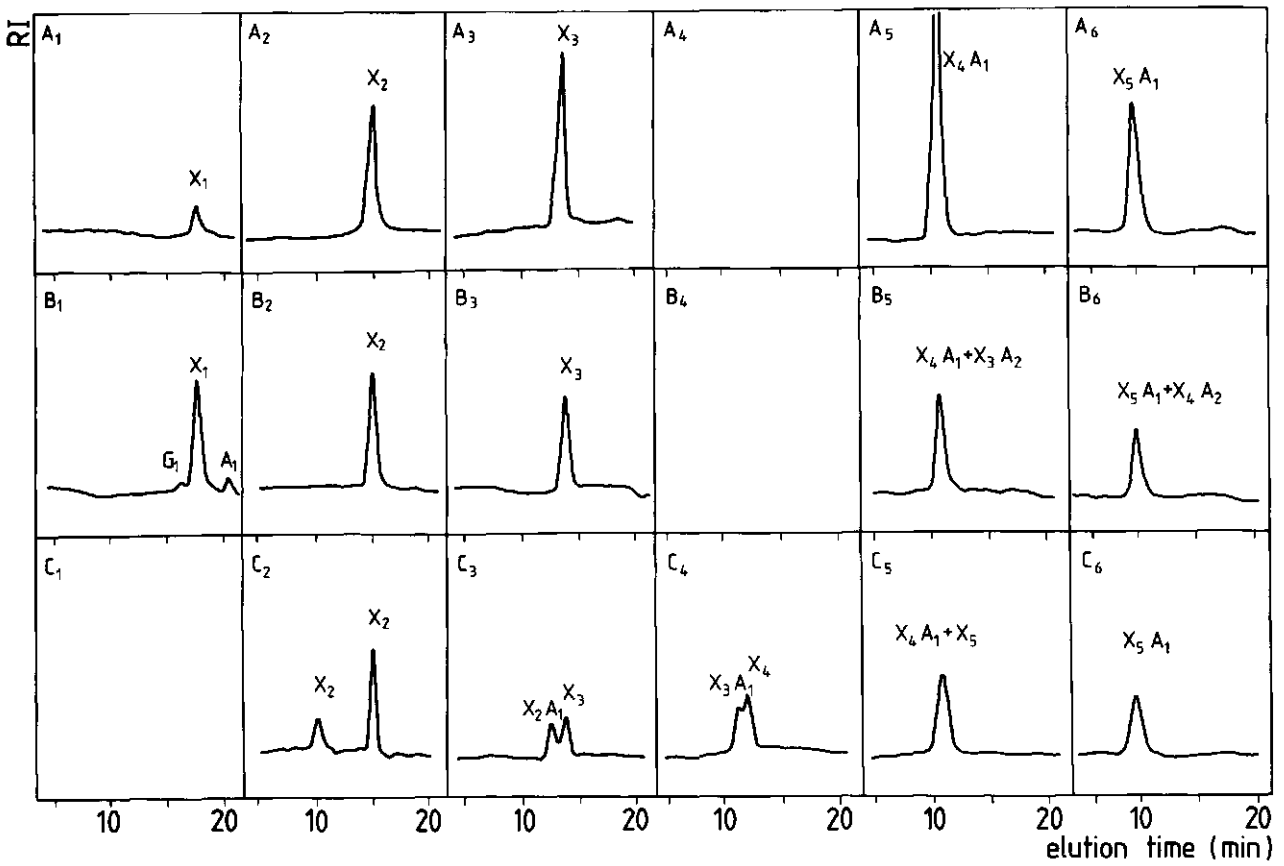

Fig. 3. HPLC analysis of mono- and oligomer fractions obtained by Bio-Gel P2 chromatography of xylan digests of Endo IV (A_1-A_6) Exo I (B_1-B_6) and β-gluc I (C_1-C_6). Monomer: A_1, B_1, C_1; Dimer A_2, B_2, C_2; Trimer A_3, B_3, C_3etc.

analysis (Fig. $3A_5$). This hetero-oligosaccharide could be identified as X_4A_1. The corresponding hexamer fractions of these digests gave a xylose to arabinose ratio of 5 on sugar analysis and single peaks at a retention time of 10.15 min. after HPLC (Fig. $3A_6$). These peaks were therefore classified as X_5A_1.

Although the penta- and hexamer peaks found after separation of the *Exo I* digest on the P2-column gave also single peaks after rechromatography on the HPLC column (Figs. $3B_5$ and $3B_6$), the sugar composition was an indication of heterogeneity. A xylose to arabinose ratio of 3 was found in the pentamer peak showing that in addition to X_4A_1, a certain amount of a compound with a higher arabinose content, probably X_3A_2 was present in this fraction. The hexamer peak gave a xylose to arabinose ratio of 4, which made us to conclude that it was a mixture of X_5A_1 and X_4A_2. The resolution of the Aminex HPX 87P column, however, may not be good enough to separate these saccharides.

Different oligosaccharides with equal molecular masses could also be demonstrated in the β-gluc I hydrolysate. The dimer fraction consisted of pure xylose and could be separated into two peaks on the HPLC column (Fig. $3C_2$). One of these peaks corresponded with the "normal" β-D-(1,4)-linked xylobiose, while the other peak at retention time of 10.26 min. probably represented the β-D-(1,3)- or β-D-(1,2)-linked isomer. The xylose to arabinose ratio of 6 in the trimer fraction and the two peaks in the HPLC chromatogram showed that this fraction consisted of X_3 and X_2A_1. From similar points of evidence the conclusion could be drawn that also the tetramer and pentamer fractions were heterogeneous and consisted of respectively $X_3A_1+X_4$ and $X_4A_1+X_5$.

Enzyme incubations with oligosaccharides

Oligosaccharides X_2, X_3, X_4A_1 and X_5A_1, which were isolated from the xylan digest of Endo IV were reincubated with the xylanolytic cellulases. The compositions of the degradation products are presented in Figure 4. As could be expected these oligosaccharides were not further degraded by Endo IV. Endo V was not active on X_2. However, X_3 was converted to a wide range of xylo-oligosaccharides, demonstrating transferase activity of this enzyme. The X_4A_1 and X_5A_1 saccharides were not, or only very scarcely attacked by this enzyme. Endo VI appeared to be active on the short-chain saccharides: X_2 and X_3 were largely degraded to the monomer. This enzyme was not active on X_4A_1, but from X_5A_1 some xylose was released. The main peak of X_5A_1 with a

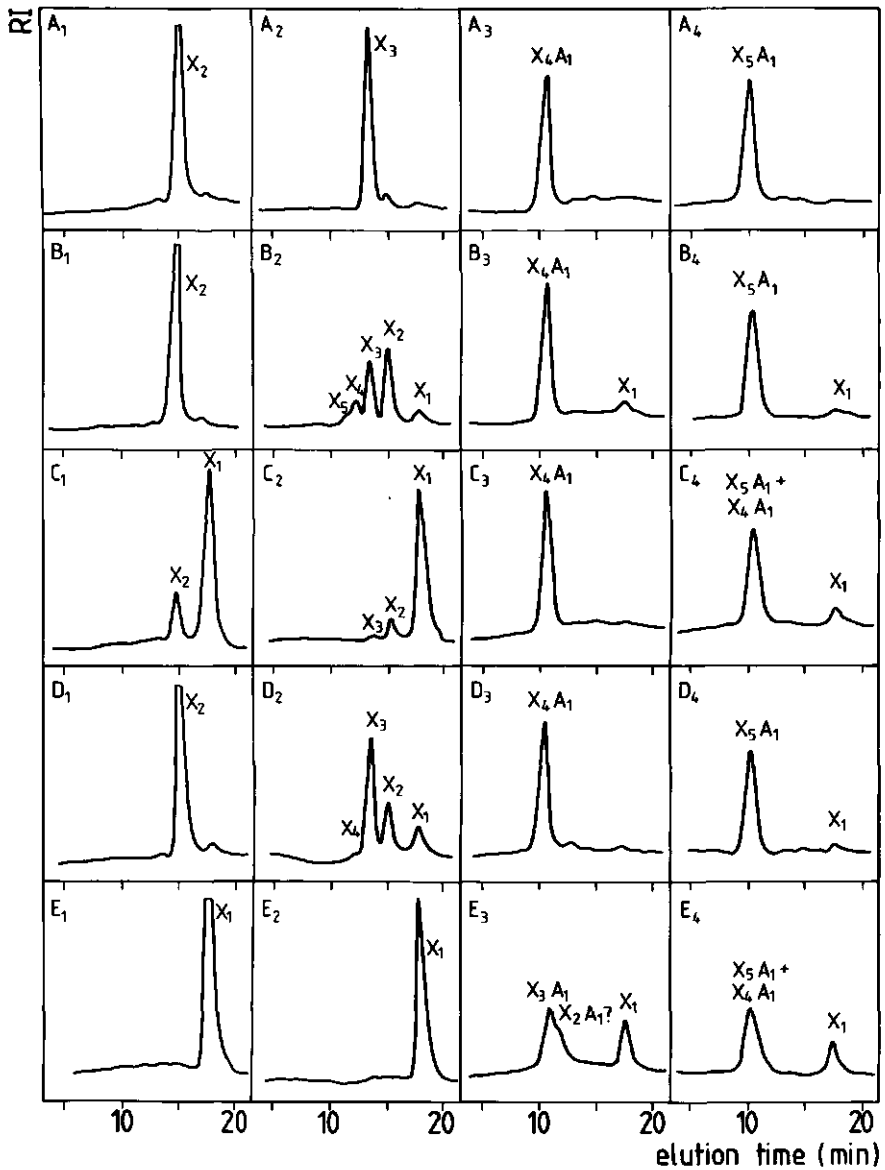

Fig. 4. HPLC analysis of products obtained from incubations of X_2, X_3, X_4A_1 and X_5A_1 with Endo IV (A_1-A_4), Endo V (B_1-B_4), Endo VI (C_1-C_4), Exo I (D_1-D_4) and β-gluc I (E_1- E_4). Products from X_2: A_1-E_1; X_3: A_2-E_2; X_4A_1: A_3-E_3 and X_5A_1: A_4- E_4.

retention time of 10.73 min. shifted to 10.55 min., demonstrating the formation of X_4A_1. Exo I showed a xylanase activity similar to Endo V: X_2 was not degraded but X_3 could be attacked by this enzyme. The formation of some X_4 displayed its transferase activity. Although Exo I liberated A_1 from polymeric xylan, surprisingly no such activity was found towards the oligomers X_4A_1 and X_5A_1. β-gluc I was highly active towards, the xylo- as well as arabinoxylo-oligosaccharides, liberating xylose as hydrolysis product. Because resolution of the separation of oligomers was not high enough, the shoulder on the X_3A_1 peak (Fig. $4E_3$) could not unambiguously be identified as X_2A_1.

Kinetic experiments

In order to investigate the specificity of the xylanolytic cellulases for xylan hydrolysis, the kinetic parameters of these enzymes were determined using xylan, as well as CM-cellulose as substrates. This latter substrate was choosen because it is, like xylan, more or less soluble. Since CM-cellulose is an unsuitable substrate for exoglucanase and β-glucosidase activity measurements, p-nitrophenyl-β-D-glucopyranoside (pNp-Gluc) was used to determine the kinetic values of Exo I and β-gluc I. The value of K_m was expressed as the (anhydro)-glycosidic substrate concentration (mM). K_{cat} was calculated from V_{max}, using the molecular masses of the enzymes as determined previously (7). Table IV shows that Endo IV, V and VI strongly prefer CM-cellulose over xylan. Compared with CM-cellulose, a much higher concentration of xylan is needed in order to saturate these enzymes to attain maximal reaction rates. Endo IV was 70 times more specific for CM-cellulose than for xylan, mainly determined by differences in K_{cat}. Endo V and Endo VI were respectively 20 and 17 times more specific for CM-cellulose.

Comparison of kinetic values of Exo I and β-gluc I on xylan and pNp-Gluc are difficult to make, because of the different natures of these substrates. However, Exo I has a very low K_m value for pNp-Gluc and was 665 times more specific for this latter substrate than for xylan, indicating its preference for β-D-glucosidic linkages. β-gluc I did not show such a strong substrate preference. The relatively much larger K_m of β-gluc I for pNp-Gluc, as well as its activity towards cellulose and xylan demonstrate the non-specific nature of this enzyme.

Tabel IV. Kinetic parameters K_m (mM glycosidic bonds), V_{max} (μmol mg.min^{-1}), K_{cat} (sec^{-1}) and K_{cat}/K_m (mM^{-1} sec^{-1}) of purified cellulases from Trichoderma viride. pNp-Gluc, p-nitrophenyl β-D-glucopyranoside; -, not active; P, Positive but not determined.

Enzyme	Parameter	Substrate		
		CM-cellulose	Xylan	pNp-Gluc
Endo I	K_m	23.7	-	-
	V_{max}	22.8		
	K_{cat}	19.0		
	K_{cat}/K_m	0.80		
Endo II	K_m	45.3	-	-
	V_{max}	42.6		
	K_{cat}	32.0		
	K_{cat}/K_m	0.71		
Endo III	K_m	2.8	-	-
	V_{max}	3.6		
	K_{cat}	3.5		
	K_{cat}/K_m	1.25		
Endo IV	K_m	25.2	106.1	-
	V_{max}	13.5	0.69	
	K_{cat}	5.3	0.3	
	K_{cat}/K_m	0.21	0.003	
Endo V	K_m	46.1	153.0	-
	V_{max}	28.8	4.63	
	K_{cat}	27.4	4.4	
	K_{cat}/K_m	0.59	0.029	
Endo VI	K_m	51.0	149.2	-
	V_{max}	31.6	5.41	
	K_{cat}	27.4	4.7	
	K_{cat}/K_m	0.54	0.032	
Exo I	K_m	P	15.2	0.11
	V_{max}		0.49	2.17
	K_{cat}		0.4	1.9
	K_{cat}/K_m		0.026	17.3
Exo II		P	-	-
Exo III		P	-	-
β-gluc I	K_m	P	25.7	21.7
	V_{max}		0.58	6.79
	K_{cat}		0.7	8.6
	K_{cat}/K_m		0.027	0.4

DISCUSSION

The cellulase complex of Trichoderma viride, which was previously shown to contain at least six endoglucanases, three exoglucanases and a β-glucosidase (7) could be subdivided into specific glucanases and non-specific glucanases. Endo I, Endo II

and Endo III, as well as Exo II and Exo III were not active on xylan, even after a prolonged incubation time. Therefore these cellulases were classified as specific glucanases. The nature of the other endoglucanases (Endo IV, Endo V and Endo VI), exoglucanase (Exo I) and β-glucosidase (β-gluc I) were less specific because these enzymes showed also activity towards xylan.

According to several authors (4, 5, 27) the group of xylan degrading enzymes can be divided into β-xylosidases, which produce xylose from short xylooligosaccharides, exo-β-xylanases, which are active on xylan and longer xylo-oligosaccharides and liberate xylose from the non-reducing end and endo-β-xylanases, which attack xylan more or less at random. This last group can be subdivided into endoxylanases which attack both long and short xylo-oligosaccharides and those which only hydrolyse longer chains. Also several highly purified xylanase preparations appear to be capable of cleaving (1,3)-α-L-arabinofuranosyl branch points from xylan. The results presented in this paper show that the purified xylanolytic cellulases fit this classification. Endo IV produced mainly X_2, X_3, X_4A_1 and X_5A_1 from xylan and was not able to release L-arabinose. Small xylo-oligosaccharides were not attacked. This enzyme acted endo-wise and preferred xylan or long xylo-oligosaccharides.

Endo V and Endo VI were also endoenzymes with respect to their xylanase activity producing a wide variety of oligosaccharides from xylan. However, these enzymes were also able to attack smaller xylo-oligosaccharides. The smallest oligosaccharide which could serve as substrate for Endo V was X_3. This endo-acting enzyme showed transferase activity with X_3 as substrate, which resulted in the formation of oligosaccharides ranging from X_1 to X_5. Similar trans-xylosidase activity was also found for an endoxylanase isolated from Bacillus pumilus (28). Endo VI could hydrolyse xylobiose as the smallest saccharide, however its activity was only moderate compared with the activity of β-gluc I towards this substrate. With respect to its xylan-degrading properties, this latter enzyme is therefore classified as a β-xylosidase.

Xylose, but no L-arabinose was liberated from X_4A_1 and X_5A_1, indicating that at least a considerable part of the L-arabinosyl branches in these oligosaccharides were attached to the non-terminal D-xylopyranosyl residues. One of the uncommon degradation products of xylan after incubation with β-gluc I was β-D-(1,3) or β-O-(1,2)-linked xylobiose. The appearance of this

oligosaccharide makes it likely that besides of L-arabinosyl branches also D-xylosyl branches exist in xylan ex oat spelts. This result is in agreement with the methylation analysis of the substrate, presented in Table I.

Exo I was found to be the only enzyme purified by us, which was able to release L-arabinose from xylan. This enzyme hydrolysed xylan by an endo-wise mechanism comparable to Endo V. Xylotriose was the smallest oligosaccharide on which activity was observed. Xylotetraose was formed as the result of trans-xylosidase activity of this enzyme. Surprisingly no L-arabinose was liberated from X_4A_1 or X_5A_1 by Exo I. Probably the enzyme needs a longer sequence of D-xylopyranosyl residues to bring the catalytic site for arabinofuranosidase activity into the vicinity of the branch point.

The results from kinetic measurements showed that the non-specific glucanases Endo IV, Endo V, Endo VI and Exo I strongly prefer β-1,4-D-glucosidic bonds over β-1,4-D-xylosidic linkages. It may be possible that these enzymes are similar to the non-specific cellulase isolated by Shikata and Nisizawa (8) who showed that CM-cellulose, xylan and p-nitrophenyl β-D-glucoside are hydrolysed at the same active site of the enzyme. The products of cellulose hydrolysis by Endo IV are glucose (G_1), cellobiose (G_2) and cellotriose (G_3) (21), while X_3 and X_2 are the main products from xylose. Similar product differences were observed for Endo V, Endo VI and Exo I. The enzymes produce G_2 and G_1 from cellulose while X_3, X_2 and X_1 are found in the xylan hydrolysates of these enzymes. If hydrolysis of both substrates takes place at the same catalytic site, formation of different oligomers can only be explained by different interactions of xylan and cellulose with the binding subsites of the enzyme, caused by structural differences of these substrates.

The nature of β-gluc I is not quite defined, because it showed no clear substrate preference. It may be possible that β-gluc I, although electrophoretically homogeneous, is a complex of saccharidases, which can only be separated under strong denaturating conditions, as described by Sprey and Lambert (20). Its fairly high molecular mass of 76000, as determined by SDS-gel electrophoresis (7) could allow for this possibility.

NOMENCLATURE

K_m	Michaelis-Menten constant (mM)
V_{max}	maximal reaction rate (μmol/mg.min)
K_{cat}	molar activity (sec^{-1})

REFERENCES

1. Linko, M., in Advances in Biochemical Engineering (Ghose, T.K. and Blakebrough, N. eds) 1977, 5, 25-48
2. Ryu, D.D.Y. and Mandels, M., Enzyme Microb. Technol. 1980, 2, 91-102
3. Bisaria, V.S. and Ghose, T.K., Enzyme Microb. Technol. 1981, 3, 90-104
4. Frederick, M.M., Kiang, C.H., Frederick, J.R. and Reilly, P.J., Biotechnol. Bioeng. 1985, 27, 525-532
5. Biely, P., Trends Biotechnol. 1985, 3, 286-290
6. Jeffries, T.W., Trends Biotechnol. 1985, 3, 208-212
7. Beldman, G. Searle-van Leeuwen, M.F., Rombouts, F.M. and Voragen, A.G.J., Eur. J. Biochem. 1985, 146, 301-308
8. Shikata, S. and Nisizawa, K.J., Biochem. 1975, 78, 499-512
9. Kubo, K. and Nisizawa, K.J., Ferment. Technol. 1983, 61, 383-389
10. Schoemaker, S.P. and Brown, R.D., Biochem. Biophys. Acta 1978, 523, 133-146
11. Wood, T.M., McCrae, S.I. and Macfarlane, C.C., Biochem. J. 1980, 189, 51-65
12. Eriksson, K.E. and Pettersson, B., Eur. J. Biochem. 1975, 51, 193-206
13. Wood, T.M. and McCrae, S.I., Biochem. J. 1978, 171, 61-72
14. McHale, A. and Coughlan, M.P., FEBS Lett. 1980, 117, 319-322
15. Sinner, M. and Dietrichs, H.H., Holzforschung 1975, 29, 168-177
16. Biely, P., Vrsanka, M. and Gorbacheva, I.V., Biochem. Biophys. Acta 1983, 743, 155-161
17. Frederick, M.M., Frederick, J.R., Fratzke, A.R. and Reilly, P.J., Carbohydr. Res. 1981, 97, 87-103
18. Shei, J.C., Fratzje, A.R., Frederucj, M.M., Frederick, J.R. and Reilly, P.J., Biotechnol. Bioeng. 1985, 27, 533-538
19. Fournier. R., Frederick, M.M. Frederick, J.R. and Reilly, P.J., Biotechnol. Bioeng. 1985, 27, 539-546
20. Sprey, B. and Lambert, C., FEMS Microbiol. Lett. 1983, 18, 217-222
21. Beldman, G. Voragen, A.G.J., Rombouts, F.M. Searle-van Leeuwen, M.F. and Pilnik, W., accepted for publication in Biotechn. Bioeng. 1986
22. Beldman, G., Voragen, A.G.J., Rombouts, F.M. and Pilnik, W., submitted for publication in Biotechn. Bioeng. 1986
23. Talmadge, K.W., Keegstra, K., Bauer, W.D. and Albersheim, P., Plant Physiol. 1973, 51, 158-173
24. Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, F.A. and Smith, F., Anal. Chem. 1956, 28, 350-356
25. Jones, T.M. and Albersheim, P., Plant Physiol. 1972, 49, 926-936
26. Somogyi, M.J., Biol. Chem. 1952, 195, 19-23
27. Dekker, R.F.H. and Richards, G.N., Adv. Carbohydr. Chem. Biochem. 1976, 32, 277-352
28. Panbangred, W., Shinmyo, A., Kinoshita, S. and Okada, H., Agric. Biol. Chem. 1983, 47, 957-963

Summary

The aim of the work presented in this thesis was to apply cellulases from Trichoderma viride for the bioconversion of agricultrual residues, as well as to study the mode of action of the cellulase complex of this fungus. These aims are specified in Chapter 1.

Chapter 2 describes the results obtained after treatment of beet pulp and potato fibre with cellulases from T. viride and pectinase derived from Aspergillus niger. It appeared that polygalacturonase from the pectinase preparation was largely responsible for the disintegration (liquefaction) of these materials. Addition of cellulases increased the rate of liquefaction. A combination of these enzymes was able to hydrolyse the cell wall polysaccharides of these substrates extensively. Enzymatic hydrolysis of beet pulp could conviently be carried out in a packed-column reactor which was percolated with an enzyme solution and from which the products were collected through an ultrafiltration membrane. The enzymes appeared to be stable in this type of reactor and could essentially be reused. Part of the enzyme activity was lost by adsorption onto the substrate residue.

The relatively high amount of lignin, present in spent grain (the residue of malt, after liquefaction and saccharification of the starch) makes the polysaccharides in this material resistant to attack of hydrolases. In Chapter 3 the effect of some physical and chemical pretreatments on the enzymatic hydrolysis of spent grain is described. Physical pretreatments which transform only the macro-structure of the substrate improved the enzymatic hydrolysis only slightly. However, pretreatment with sulphuric acid or sodium hydroxide (0.5 N) doubled the degree of polysaccharide degradation after enzymic treatment. The mono- and oligosaccharide composition of the digest was depedent on the pretreatment method. Predominantly monosaccharides were detected in the enzymic product of the acid pretreated spent grain. On the other hand a large amount of oligosaccharides was present in the digest of the alkali pretreated material.

In order to investigate the mode of action of the cellulase complex, all detectable endoglucanase, exoglucanase and β-glucosidase activities were isolated from a commercial cellulase

preparation, derived from T. viride (Chapter 4). Six endoglucanases (Endo I; II; III; IV; V; VI), three exoglucanases (Exo I; II; III) and a β-glucosidase (β-gluc I) were isolated and characterized with respect to their activities on several substrates and some chemical and physical properties. The endoglucanases Endo I, II and IV were more random in their attack on carboxymethyl cellulose than the other endoglucanases. The exoglucanases produced cellobiose from H_3PO_4-swollen cellulose, however, only Exo I was also able to hydrolyse this product to glucose. β-Gluc I was highly active towards p-nitrophenyl-β-D-glucose but not towards cellobiose.

The adsorption behaviours of the purified endoglucanases and exoglucanases on crystalline cellulose are described in Chapter 5. Endo I, III and V adsorbed much stronger on this type of cellulose than the other endoglucanases. With respect to this behaviour also the exoglucanases were subdivided: Exo III adsorbed strongly and Exo II only moderately. Adsorption was only partially reversible. Kinetic measurements indicated that a part of the adsorbed enzyme molecules are not active. It is supposed that these enzymes (for instance an endoglucanase) express their hydrolytic activity only when an other enzyme (i.e. an exoglucanase) is also present. This might explain the synergism, often observed during cooperative hydrolysis of crystalline cellulose by endoglucanases and exoglucanases.

A more detailed investigation on synergism is described in Chapter 6. It appeared that the degree of synergistic effect was largely dependent on the initial ratio of endoglucanase to exoglucanase and maximal at a specific value. The stronger an endoglucanase adsorbs at its substrate, the less is needed of this enzyme to reach a maximal degree of synergism, when working in concert with an exoglucanase.

The specific and non-specific nature of the purified glucanases with regard to their activity on xylan and cellulosic substrates is presented in Chapter 7. Endo I, II and III as well as Exo II and III could be classified as specific glucanases. Endo IV, V, VI and Exo I were able to degrade xylan, however these enzymes clearly preferred to hydrolyse β-1,4-glucosidic linages. β-Gluc I showed no clear substrate preference.

Samenvatting

Koolhydraten komen op de wereld in overvloed voor en kunnen gebruikt worden voor de productie van levensmiddelen of als grondstof voor de chemische of fermentatie-industrie. Helaas is de natieve verschijningsvorm van de meeste van deze stoffen niet zodanig dat gebruik direct mogelijk is. Zo moet bijvoorbeeld zetmeel (het polymeer van α-D-glucose), dat in een semi-kristallijne vorm als korrels in de natuur aanwezig is, eerst door een hittebehandeling worden ontsloten en vervolgens met enzymen worden vervloeid en versuikerd om een fermenteerbare suikeroplossing te verkrijgen. Ongeveeer 40 % van de totale plantaardige biomassa op aarde bestaat uit een ander polymeer van glucose, namelijk cellulose. Echter in deze stof zijn de glucose-eenheden via een β-(1,4)-glucosidische verbinding gekoppeld, waardoor de ketens een structuur krijgen die sterke onderlinge interactie, kristallijn van aard, mogelijk maakt. Met behulp van cellulases zou cellulose benut kunnen worden, door omzetting tot glucose. Het werk beschreven in dit proefschrift had tot doel commerciële cellulases, afkomstig van de schimmel Trichoderma viride te gebruiken voor de biologische omzetting van enkele plantaardige residuen en daarnaast het werkingsmechanisme van het gebruikte cellulase-complex te bestuderen. Deze doelstellingen zijn nader uitgewerkt in hoofdstuk 1.

Hoofdstuk 2 beschrijft de resultaten die werden verkregen bij behandeling van bietenpulp en aardappelvezel met cellulase afkomstig van T. viride en pectinase geproduceerd door Aspergillus niger. Het bleek dat het enzyme polygalacturonase, aanwezig in het pectinase preparaat, verantwoordelijk was voor een snelle desintegratie (vervloeiing) van het materiaal. Dit effect kon worden versterkt door toevoeging van cellulases. Een combinatie van beide enzympreparaten was in staat de celwandpolysachariden van de genoemde substraten verregaand te hydrolyseren. Bietenpulp kon op een eenvoudige manier enzymatisch omgezet worden door gebruik te maken van een met substraat gepakte kolom, waardoor een enzymoplossing werd gepompt en waaruit het product door een ultrafiltratiemembraan werd verwijderd. De enzymen bleven stabiel in een dergelijk systeem en zouden in principe opnieuw gebruikt kunnen worden. Een gedeelte van de activiteit ging verloren door adsorptie aan substraat residue.

Bierbostel, het residue van mout na vervloeiing en versuikering van het zetmeel, bevat relatief veel lignine, dat een barriere vormt voor de polysacharide-afbrekende enzymen. In hoofdstuk 3 wordt beschreven wat het effect van enkele mechanische en chemische voorbehandelingen is op de enzymatische afbreekbaarheid van bierbostel. Mechanische voorbehandelingen, die alleen de macro-structuur veranderen bleken de enzymatische polysacharide-afbraak slechts in geringe mate positief te beinvloeden. Daarentegen kon door voorbehandeling met zwavelzuur of natronloog en nabehandeling met een combinatie van cellulases de polysacharide-afbraak worden verdubbeld. De mono- en oligosacharidesamenstelling van het verkregen product was sterk afhankelijk van de gebruikte voorbehandeling. In het enzymatische product van het met zuur voorbehandelde materiaal werden voornamelijk monosachariden gevonden, terwijl na alkalische voorbehandeling nog veel oligosachariden werden aangetoond.

Om het werkingsmechanisme van het cellulasecomplex nader te kunnen bestuderen is het een eerste vereiste de beschikking te hebben over gezuiverde en goed omschreven cellulases. Een procedure om alle meetbare endoglucanase-, exoglucanase- en β-glucosidase-activiteiten te zuiveren uit een mengsel van cellulases, aanwezig in een commercieel enzympreparaat afkomstig van T. viride, staat beschreven in hoofdstuk 4. Er konden zes endoglucanases (Endo I; II; III; IV; V; VI), drie exoglucanases (Exo I; II; III) en een β-glucosidase (β-gluc I) worden geisoleerd. Deze enzymen werden nader gekarakteriseerd met betrekking tot hun activiteit op diverse substraten en enkele chemische en fysische eigenschappen. De manier waarop de endoglucanases Endo I, II en IV carboxymethylcellulose hydrolyseren is meer willekeurig van aard dan de overige endoglucanases. De drie exoglucanases bleken cellobiose te produceren van fosforzuur-gezwollen cellulose, maar alleen Exo I was in staat dit product verder te hydrolyseren tot twee glucose eenheden. β-Gluc I, dat zeer actief was op p-nitrophenyl-β-D-glucoside, was daarentegen niet actief op cellobiose. In dit hoofdstuk wordt tevens ingegaan op de rol die de cellobiohydrolases (Exo II en III) spelen tijdens de afbraak van kristallijn cellulose. De resultaten suggereren dat de cellobiohydrolases met name actief zijn op nieuw gegenereerde ketenuiteinden.

Het adsorptiegedrag van de diverse gezuiverde endoglucanases en exoglucanases ten opzichte van kristallijn cellulose was verschillend van aard. Uit de resultaten, vermeld in hoofdstuk 5, bleek dat de endoglucanases Endo I, III en V sterk

adsorbeerden aan dit type cellulose, in tegenstelling tot een andere groep endoglucanases, bestaande uit Endo II, IV en VI, die slechts zeer matig adsorbeerden. Eenzelfde onderscheid kon gemaakt worden voor cellobiohydrolases Exo II en Exo II, waarvan Exo III duidelijk sterker adsorbeerde. De adsorptie was slechts gedeeltelijk reversibel. Kinetische experimenten gaven een aanwijzing dat een gedeelte van de geadsorbeerde enzymmolekulen niet in staat zijn hun hydrolytische eigenshap tot expressie te brengen. Mogelijkerwijs komt de activiteit van deze enzymen (bijvoorbeeld een endoglucanase) alleen dan tot uiting als een ander enzym (in dit geval een exoglucanases) ook aanwezig is in de directe omgeving. Dit zou een verklaring kunnen zijn voor het synergistisch effect dat over het algemeen optreedt als een endoglucanase en exoglucanase gezamenlijk inwerken op kristallijn cellulose.

Nader onderzoek naar het synergisme tussen endoglucanases en exoglucanases staat beschreven in hoofdstuk 6. De mate van synergistisch effect bleek sterk af te hangen van de initiele verhouding waarin beide enzymen gebruikt werden en was maximaal bij een bepaalde waarde. Naarmate een endoglucanase beter adsorbeert bleek er relatief minder van dit enzym toegevoegd hoeven te worden om het maximale synergistische effect met een exoglucanase te bereiken.

In hoofdstuk 7 wordt de activiteit van de gezuiverde cellulases ten opzichte van xylaan beschreven. Endo I, II en III, maar ook Exo II en III vertoonden geen activiteit op xylaan en werden geclassificeerd als specifieke glucanases. Endo IV, V en VI alsmede Exo I en β-gluc I bleken minder specifiek van aard en waren in staat xylaan te hydrolyseren volgens een patroon dat overeenkomt met het hydrolytische karakter van specifieke xylanases. Kinetische experimenten met verschillende substraten toonden aan dat de niet-specifieke endoglucanases en exoglucanase Exo I bij voorkeur β-1,4-glucosidische verbindingen hydrolyseren. Het geisoleerde β-glucosidase had een minder specifieke substraatvoorkeur.

Curriculum vitae

Gerrit Beldman werd op 2 juni 1955 te Zutphen geboren. Na het behalen van zijn diploma HBS-B, studeerde hij scheikunde gedurende de periode van 1972 tot 1980 aan de Rijks Universiteit te Groningen. Na zijn kandidaatsexamen werd als hoofdvak biochemie en als bijvak radiobiologie gedaan. Het promotie-onderzoek vond plaats van 1980 tot 1984 op het Laboratorium voor Levensmiddelen chemie en -microbiologie van de Landbouw Hogeschool te Wageningen. Na deze periode was hij op ditzelfde laboratorium enige tijd werkzaam als tijdelijk wetenschappelijk medewerker. Sinds begin 1985 is hij werkzaam bij het NIKO-TNO te Groningen.