Biochem. J. (1980) **191**, 863–866
Printed in Great Britain

The adaptability, purification and properties of exo-β1,3-glucanase from the fungus *Trichoderma reesei*

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(*Received 18 August 1980/Accepted 27 August 1980*)

The fungus *Trichoderma reesei* grows on barley (*Hordeum valgare* L.) β-glucan and pachyman, secreting increased quantities of exo-β1,3-glucanase. This enzyme is also found in commercial cellulase preparations, from which it has been partially purified. It has a mol.wt. of 70000, an isoelectric point of 4.2, is cold-stable and hydrolyses both β1→3- and β1→6-linkages.

Trichoderma reesei [formerly *T. viride*; Simmons (1977)] is probably the most widely investigated cellulolytic fungus and the enzymes comprising its cellulase complex have been extensively studied (Pettersson *et al.*, 1972). Nevertheless, there has been little commercial application of cellulases (Aunstrup, 1978). However advantage has been taken of the ability of *T. reesei* cellulase to hydrolyse β-glucan from barley (*Hordeum vulgare* L.). This glucan contains 70% β1→4- and 30% β1→3-glucosidic linkages (Bathgate & Dalgliesh, 1974) and causes serious problems if it accumulates during the brewing process. *T. reesei* cellulase has been used to relieve these problems (Stentebjerg-Olesen, 1980) and is also used in the determination of barley β-glucan (Bamforth *et al.*, 1979; Martin & Bamforth, 1980, 1981).

Although much is known of the β1,4-glucanases produced by *T. reesei*, little is known of the β1,3-glucanases. Of a wide range of microorganisms, *T. reesei* was shown to be richest in apparently constitutive β1,3-glucanase (Chesters & Bull, 1963*a*). This activity could be divided into a major exo-enzyme (EC 3.2.1.58) and a minor endo-component (EC 3.2.1.39) (Chesters & Bull, 1963*b*). There has not previously been a detailed investigation of the β1,3-glucanases of *T. reesei*. In the present paper endo- and exo-β1,3-glucanases are demonstrated in commercial *T. reesei* cellulase preparations and a detailed study of the exo-β1,3-glucanase is reported. Additionally the adaptability of this and other enzymes during growth of *T. reesei* on various glucans is discussed.

Experimental

Materials

All reagents were of highest available purity. Barley β-glucan was from Biocon, Tenbury Wells, Worcs., U.K. Crystalline laminarin and sodium carboxymethylcellulose (medium viscosity) were from Sigma, Poole, Dorset, U.K. Pachyman was from Sam-Ae Trading Co., Seoul, South Korea, and was converted into the carboxymethyl derivative (sodium salt) as described by Clarke & Stone (1962). Pustulan was a gift of Dr. R. Stark, Heriot–Watt University, Edinburgh, Scotland, U.K. Cellulase, a freeze-dried culture medium from *T. reesei* strain QM 6a (A.T.C.C. 26801) grown on bran, was from Worthington Enzymes (Millipore, London NW10 7SP, U.K.). Solutions of cellulase were heated and dialysed before use (Bamforth *et al.*, 1979).

T. reesei strain IMI 84562 was from the Commonwealth Mycological Institute, Kew, Surrey, U.K., and was maintained on slopes of potato-dextrose/agar.

Enzyme assays

Laminarinase (*exo-β1,3-glucanase*). Laminarin (0.11 mg), enzyme and 50 mM-sodium succinate, pH 5.5, were mixed in a final volume of 1 ml. Reaction at 40°C was started by adding enzyme and terminated after 30 min by boiling (1 min). Glucose or reducing sugar was measured. As controls, laminarin or enzyme were omitted or boiled enzyme was used.

Barley exo-β-glucanase. This was assayed similarly, except that laminarin was replaced by barley β-glucan (0.14 mg).

Carboxymethylcellulase (*exo-β1,4-glucanase*). Carboxymethylcellulose (2 mg) replaced laminarin in the above assay.

Endo-β1,3-glucanase. This was determined by using 0.8% (w/v) sodium carboxymethylpachyman (3 ml) and enzyme in a total volume of 3.5 ml of

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50mM-succinate, pH5.5. After 30min at 35°C reaction was stopped by adding 0.1M-$HgCl_2$ (0.1ml) and the viscosity measured by using miniature U-tube viscometers, size M2 (Scientific Supplies Ltd., London E.C.1, U.K.). Control assays contained no enzyme. Endo-β1,4-glucanase (EC 3.2.1.4) and barley endo-β-glucanase were measured similarly, except that the substrates were 0.2% (w/v) sodium carboxymethylcellulose and 0.4% (w/v) barley β-glucan respectively.

Enzyme units

One unit of an exo-enzyme is defined as the quantity that catalyses the formation of 1μg of glucose/min. One unit of an endo-enzyme is the amount that causes a 10% reduction in substrate specific viscosity in 100min.

Chemical determinations

Glucose was measured by using the glucose oxidase/peroxidase kit of Boehringer, Lewes, Sussex, U.K. (catalogue no. 124 036). Reducing sugars were assayed by the method of Somogyi (1952), glucose being used for calibration. Protein was determined by the Kalb & Bernlohr (1977) method.

Isoelectric focusing

An LKB Ampholine 8100-2 column was used. Ampholines pH3.5–10 (1%) and glycerol stabilization, with the anode at the tope of the column, were used. Heated cellulase (4ml) was mixed with the light gradient solution. Focusing, at 20°C, was at 10W and was continued until minimum current flowed. Collected fractions were, after pH determination, dialysed for 24h at 4°C against 50mM-potassium phosphate, pH6.5, before measurement of laminarinase activity.

Partial purification of exo-β1,3-glucanase

Step one: salt fractionation. Heat-treated cellulase was adjusted to 40% saturation with $(NH_4)_2SO_4$ at 4°C. After being stirred at 0°C for 20min, the mixture was centrifuged (25000**g**, 10min, 0°C). The supernatant was taken successively from 40–60% to 60–80% and 80–90% saturation by adding solid $(NH_4)_2SO_4$, and the precipitate was removed at each stage. These were re-dissolved in 2ml each of 50mM-potassium phosphate, pH7.0. Of the β1,3-glucanase recovered, 60% was present in the fraction precipitated at 60–80% saturation.

Step two: gel-permeation chromatography. The redissolved pellet was applied to a column (2cm × 31cm) of Bio-Gel P-150 equilibrated with 50mM-potassium phosphate, pH7.0. The column was eluted (12ml/h) with the same buffer in a descending mode at 20°C. Collected fractions (2ml) were assayed for laminarinase and protein. Those showing laminarinase activity were pooled and stored at 4°C.

Polyacrylamide-gel electrophoresis. This was performed by the method of Davis (1964), except that the large-pore gel was omitted. Electrophoresis was performed in a Shandon (London N.W.10, U.K.) apparatus with 7% (w/v) gels at pH8.3 with Bromophenol Blue as tracking dye. Protein was stained by the method of Blakesley & Boezi (1977). R_F values are quoted relative to the mobility of Bromophenol Blue.

Results

Hydrolysis of barley β-glucan (152μg) by heat-treated cellulase (300μg of protein) formed 164μg of glucose, i.e. a 97% recovery. Glucose is adsorbed by cellulase (Martin & Bamforth, 1981) and this accounts for the small deficit in glucose recovery. Complete hydrolysis of barley β-glucan requires that cellulase contains β1,3-glucanase. Heat-treated cellulase preparations release reducing sugar from laminarin. The initial rate of release of reducing sugar is slightly greater than that of glucose, and it was estimated that 76% of the total β1,3-glucanase activity is due to an exo-enzyme and the remainder is endo-acting. The exo-enzyme has been studied in detail.

The specific activity of laminarinase in heat-treated cellulase is 38.4 units/mg of protein. Initial rates of glucose formation are proportional to assay time and to protein concentration below 174μg/assay. Heating cellulase at 60°C for 10min, which is necessary to destroy amyloglucosidase activity (Bamforth *et al.*, 1979), leads to the loss of 64% of the laminarinase activity but no loss of exo-β1,4-glucanase activity.

Properties of exo-β1,3-glucanase

Enzyme purified as described in the Experimental section had a specific activity of 612 units/mg of protein and the activity yield was 9%. It did not release glucose from carboxymethylcellulose or from barley β-glucan. The enzyme was not inactivated by $(NH_4)_2SO_4$ (cf. Chesters & Bull, 1963*c*). One peak of laminarinase activity (measured by formation of reducing sugar) was separated on a calibrated column of Bio-Gel P-150. The elution position of the enzyme indicated a mol.wt. of 70000. Polyacrylamide-gel electrophoresis at pH8.3 revealed one major protein band (R_F 0.20) and a minor band (R_F 0.31).

On storage for 10 days, the enzyme (46μg of protein/ml) retained 79% of its activity at −15°C and 85% at 4°C. Heating the enzyme (540μg of protein/ml) at 50 or 60°C for 15min led to the loss of 82 and 100% activity respectively, i.e. the enzyme is less stable in purified preparation.

Glucose yield from laminarin was maximal at pH 5.0–5.5 in succinate buffer. A K_m of 0.28 mg of laminarin/ml was measured. The enzyme also releases glucose from carboxymethylpachyman and pustulan.

Purified laminarinase gave identical rates of formation of glucose and reducing sugar from laminarin, indicating that it is an exo-enzyme.

Heat-treated cellulase preparations have also been fractionated by isoelectric focusing. One laminarinase peak was obtained, with pI 4.2. Laminarinase activity was measured after dialysis of fractions against phosphate buffer. In the assay, lower, but still significant, amounts of reducing sugar were released in controls from which laminarin was omitted. Boiling the enzyme did not decrease such endogenous sugar release. The phenomenon was observed only when laminarinase samples had been subjected to isoelectric focusing. Further experimentation is needed to explain these observations.

Enzyme adaptation in T. reesei

T. reesei strain IMI 84562 grows readily on glucose and barley β-glucan and even more readily on insoluble pachyman. Growth is thinner, with clumping, on carboxymethylcellulose. Low activities of exo-β1,3-glucanase are found in the medium when *T. reesei* is grown on glucose (Table 1). Somewhat higher activities are present when carboxymethylcellulose is the growth substrate. However, activity is considerably increased when either barley β-glucan or pachyman (a β1→3-glucan; Warsi & Whelan, 1957) are the carbon sources. Highest endo-β1,3-glucanase activity is present when cells are grown on pachyman, lowest when glucose is growth substrate. The overall difference in activity is only 2-fold. High activities of endo-β1,4-glucanase are present only when *T. reesei* is grown on carboxymethylcellulose. On all four substrates the organism produces similar high activities of endo-barley-β-glucanase. This seems to reflect the presence of a distinct constitutive enzyme capable of hydrolysing β-glucan. This may be a proteinase or peptidase, as barley β-glucan is covalently bound to peptide (Forrest, 1977). Carboxypeptidase is involved in solubilizing and degrading β-glucan in barley (Bamforth *et al.*, 1979; Martin & Bamforth, 1980), probably by hydrolysing peptide–glucan ester linkages involved both in cross-linking β-glucan molecules and in binding β-glucan to endosperm cell walls.

Discussion

Several uses have been suggested for purified β1,3-glucanases, including the degradation of fungal cell walls (Aunstrup, 1978) and in the study of cell-wall structure (Rombouts *et al.*, 1978). Commercial preparations of *T. reesei* cellulase constitute a convenient source of exo-β1,3-glucanase in high activity, which is readily purified and cold-stable.

Several exo-β1,3-glucanases from other organisms have been reported, and the present enzyme largely resembles them. The major difference is its higher molecular weight. Yeast exo-β1,3-glucanases have molecular weights in the range 20000–40000 (Notario *et al.*, 1976; Abd-El-Al & Phaff, 1968), whereas those of other funal laminarinases are 43000–51000 (Huotari *et al.*, 1968; Nagasaki *et al.*, 1977; Hadibi *et al.*, 1977). In common with

Table 1. *Adaptability of enzymes in T. reesei*

T. reesei strain IMI 84562 was grown on potato-dextrose/agar. After 8 days, spores were washed from the agar with sterile deionized water, washed and resuspended in growth medium (50 ml) contained in 250-ml Erlenmeyer flasks. The growth medium was that described by Reese & Mandels (1959), containing 0.5% (w/v) of glucose, carboxymethylcellulose, barley β-glucan or pachyman as substrate, all of which were soluble except for pachyman. Flasks were shaken for 14 days at 25°C. Growth medium (10 ml) was then dialysed against 50 mM-sodium succinate, pH 5.5 (1 litre) at 4°C before it was examined for enzyme content. Assays were performed in duplicate on media from two separate growth flasks per substrate.

			Specific activity (units/mg of protein)				
			Exo-β1,3-glucanase				
Growth substrate	Total protein in medium (mg)	Substrate ...	Laminarin	Carboxymethyl-pachyman	Endo-β1,3-glucanase	Endo-β1,4-glucanase	Barley endo-β-glucanase
Glucose	15.7		3	10	252	64	758
Carboxymethyl-cellulose	9.0		24	30	358	1389	2467
Barley β-glucan	12.4		91	—	470	105	1290
Pachyman	12.9		—	75	550	87	1923

yeast, but not other fungal laminarinases, the enzyme from *T. reesei* hydrolyses β1,6-glucans. The *T. reesei* enzyme, like most other laminarinases, will not hydrolyse $\beta(1\rightarrow3/1\rightarrow4)$-glucans of the type found in barley, indicating that such glucans do not have a succession of $\beta1\rightarrow3$-glucosidic linkages at their non-reducing ends.

Various roles have been suggested for β1,3-glucanases, including mobilization of intracellular food reserves, extracellular hydrolysis of plant debris and catalysis of architectural changes in fungal cell walls (Bull & Chesters, 1966). It has previously been accepted that fungal laminarinases are constitutive (Reese & Mandels, 1959) and bacterial laminarinases are inducible (Tanaka, 1964). Other fungal enzymes, e.g. cellulase, chitinase and xylanase, are adaptive (Reese & Mandels, 1959). The apparently constitutive presence of the β1,3 glucanases suggested that the principal role for these enzymes is in the hydrolysis of the cell walls of the fungus.

However, we have found that activities of exo-β1,3 glucanase are greatly increased and those of endo-β1,3-glucanase slightly increased when *T. reesei* grows on mixed-linkage β-glucan or on pachyman. Whether this reflects induction by the polymers (presumably through the action of their hydrolysis products) *per se* or catabolite repression by the more favoured growth substrate, glucose, cannot be deduced from these results. Del Rey *et al.* (1979) showed that glucose represses β1,3-glucanase synthesis in several organisms, but not in *T. reesei* or baker's yeast, *Saccharomyces cerevisiae*. This suggests that exo-β1,3-glucanase in *T. reesei* is inducible, and we conclude that this enzyme is synthesized by *T. reesei* in response to the presence of food sources containing $\beta1\rightarrow3$-bonds. Mandels *et al.* (1962) found that sophorose (2-*O*-β-D-glucopyranosyl-D-glucose) is the most active inducer of *T. reesei* cellulase, showing that enzymes specific to one glucosidic-linkage type are inducible by compounds containing a different glucosidic linkage. This may account for the somewhat increased activity of laminarinase when *T. reesei* is grown on carboxymethylcellulose.

Elucidation as to which other enzymes are involved in β-glucan and pachyman hydrolysis by *T. reesei* requires further study.

Miss S. Harding is thanked for assistance with the growth experiments and Dr. P. J. Large for valued advice.

References

Abd-El-Al, A. T. H. & Phaff, H. J. (1968) *Biochem. J.* **109**, 347–360

Aunstrup, K. (1978) in *Annual Reports on Fermentation Processes* (Perlman, D., ed.), vol. 2, pp. 125–154, Academic Press, London and New York

Bamforth, C. W., Martin, H. L. & Wainwright, T. (1979) *J. Inst. Brew.* **85**, 334–338

Bathgate, G. N. & Dalgliesh, C. E. (1974) *Proc. Am. Soc. Brew. Chem.* **33**, 32–36

Blakesley, R. W. & Boezi, J. A. (1977) *Anal. Biochem.* **82**, 580–582

Bull, A. T. & Chesters, C. G. C. (1966) *Adv. Enzymol. Relat. Areas. Mol. Biol.* **28**, 325–364

Chesters, C. G. C. & Bull, A. T. (1963*a*) *Biochem. J.* **86**, 28–31

Chesters, C. G. C. & Bull, A. T. (1963*b*) *Biochem. J.* **86**, 31–38

Chesters, C. G. C. & Bull, A. T. (1963*c*) *Biochem. J.* **86**, 38–46

Clarke, A. E. & Stone, B. A. (1962) *Phytochemistry* **1**, 175–188

Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427

Del Rey, F., Garcia-Acha, I. & Nombela, C. (1979) *J. Gen. Microbiol.* **110**, 83–89

Forrest, I. S. (1977) *Biochem. Soc. Trans.* **5**, 1154–1156

Hadibi, E.-H., Donati, R. & Bastide, M. (1977) *C.R. Hebd. Séances Acad. Sci. Ser. D* **283**, 1397–1399

Huotari, F. I., Nelson, T. E., Smith, F. & Kirkwood, S. (1968), *J. Biol. Chem.* **243**, 952–956

Kalb, V. F., Jr. & Bernlohr, R. W. (1977) *Anal. Biochem.* **82**, 362–371

Mandels, M., Parrish, F. W. & Reese, E. T. (1962) *J. Bacteriol.* **83**, 400–408

Martin, H. L. & Bamforth, C. W. (1980) *J. Inst. Brew.* **86**, 216–221

Martin, H. L. & Bamforth, C. W. (1981) *J. Inst. Brew.* in the press

Nagasaki, S., Saito, K. & Yamamoto, S. (1977) *Agric. Biol. Chem.* **41**, 493–502

Notario, V., Villa, T. G. & Villanueva, J. R. (1976) *Biochem. J.* **159**, 555–562

Pettersson, L. G., Axiö-Fredriksson, U. B. & Berghem, L. E. R. (1972) *Proc. Int. Ferment. Symp. 4th* 727–729

Reese, E. T. & Mandels, M. (1959) *Can. J. Microbiol.* **5**, 173–185

Rombouts, F. M., Fleet, G. H., Manners, D. J. & Phaff, H. J. (1978) *Carbohydr. Res.* **64**, 237–249

Simmons, E. G. (1977) *Int. Mycol. Cong. 2nd, Tampa* 618

Somogyi, M. (1952) *J. Biol. Chem.* **195**, 19–23

Stentebjerg-Olesen, B. (1980) *Proc. Conv. Inst. Brew., (Austr. Sect.) 16th, Sydney* in the press

Tanaka, H. (1964) Ph.D. Thesis, University of California, Davis

Warsi, S. A., & Whelan, W. J. (1957) *Chem. Ind. (London)* 1573