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A NEW TYPE OF ENZYME, AN EXO-SPLITTING α-1,3 GLUCANASE FROM NON-INDUCED CULTURES OF *ASPERGILLUS NIDULANS*

B. J. M. ZONNEVELD
Genetisch Laboratorium der Rijksuniversiteit, Leiden (The Netherlands)

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SUMMARY

1. A new type of enzyme has been isolated and characterized as an exo-splitting α-1,3 glucanase; this enzyme is produced by *Aspergillus nidulans*.

2. It is not necessary to induce the enzyme; both the substrate (α-1,3 glucan) and α-1,3 glucanase are isolated from the same organism.

3. The enzyme degrades α-1,3 glucan to glucose but is inactive on glucan with mixed α-1,3 and α-1,4 linkages (nigeran).

4. The enzyme has the following properties: K_m (g/ml) is $1.6 \cdot 10^{-3}$, the pH optimum lies between 5 and 6.2; the temperature optimum lies at 50°; it is shown to be an exo-enzyme; its activity is not inhibited by Mg^{2+}, Zn^{2+}, Cu^{2+}, EDTA or glucose.

INTRODUCTION

Biochemical analysis revealed that part of the cell wall of *Aspergillus nidulans* consists of α-1,3 glucan[1,9].

The α-1,3 glucans found in both Ascomycetes and Basidiomycetes[2] are attacked by only a few organisms. So far, only two endo-enzymes that hydrolyse glucans with α-1,3-linkages have been described. REESE AND MANDELS[3] found only six out of 166 organisms studied that could hydrolyse nigeran (α-1,3, α-1,4 linkages). The enzyme they described, called mycodextranase, does not attack pure α-1,3 glucan[1,4] and is produced, for example, by *Trichoderma viride* QM 6a. However, the same organism was used by HASEGAWA *et al.*[5] to isolate an enzyme that proved to be active on both pure α-1,3 glucan and nigeran. The enzyme from *Aspergillus nidulans* to be described here is an exo-α-1,3 glucanase that attacks α-1,3 glucan but not nigeran. This enzyme is produced without the addition of any α-1,3 glucan to the culture medium.

MATERIAL AND METHODS

Organism; culture conditions

A biotin-dependent strain (biA_1) of *Aspergillus nidulans* (Eidam) of Glasgow

origin was used throughout. Medium and culture conditions were as described by PONTECORVO[6], but the medium was supplemented with biotin and 2% glucose was used instead of 1%.

Enzyme production

For enzyme production, cultures were grown from thick conidial suspensions on agar plates (0.75% agar). Maximum enzyme production was obtained after 6 days. Plates were homogenized in a Servall-Omnimixer (immersed in an ice-bath) for 10 min after addition of the same volume of citrate–phosphate buffer (pH 6.2). The homogenate was centrifuged for 10 min at 4000 rev./min. After a second centrifugation, the clear yellowish-brown supernatant fluid was reserved for further purification.

Enzyme purification

To the supernatant 2 vol. of acetone (−18°) were added. After being held for 2 h at −18°, the precipitate was collected by centrifugation, resuspended in 10 ml citrate–phosphate buffer at 4°, and dialysed overnight against the same buffer at 4°. The precipitate was removed by centrifugation and the supernatant fluid used as a crude enzyme preparation. A 1-ml aliquot of this crude extract was brought onto a Sephadex G-100 column (15 mm × 800 mm) and fractions of 2 ml were assayed for glucanase activity. The fractions containing the exo-α-1,3 glucanase were pooled and used for characterization of the enzyme.

Enzyme assays

Aliquots of 0.9 ml (2 mg/ml) of α-1,3 glucan or 0.9 ml (1.25 mg/ml) laminarin or soluble starch were incubated with 0.1 ml of the pooled fractions and incubated for 3 h at 37°. All substrates were dissolved or suspended in citrate–phosphate buffer (pH 6.2).

Enzyme activity was measured either as an increase in reducing power with the neocuproine reagent[7] or by reacting the incubation mixtures (after removal of the remaining insoluble substrate) with the anthrone reagent[8] or with the Glucostat reagent (0.5 h at 37°) (Worthington Biochemical Company, New Jersey). One unit of enzyme is defined as the amount of enzyme liberating 1 μmole glucose per min.

Isolation of α-1,3 glucan

See for isolation of α-1,3 glucan and the purity of the substrate ZONNEVELD[9]. For enzymic degradation, the α-1,3 glucan was dissolved in 5% KOH, precipitated with glacial acetic acid, and then washed with water. Finally, the α-1,3 glucan was suspended in citrate-phosphate buffer in a concentration of 2 mg/ml.

Miscellaneous

Protein was measured with the Folin–Ciocalteau reagent[10], bovine albumin serving as a standard. For chromatography, thin-layer plates of Silica Gel were irrigated with butanol–acetone–water (4:5:1, by vol.) for 1 h and, after drying, with chloroform–acetic acid–water (10:7:1, by vol.) in the same direction for 1.5 h. The detecting reagent was analine hydrogenphtalate.

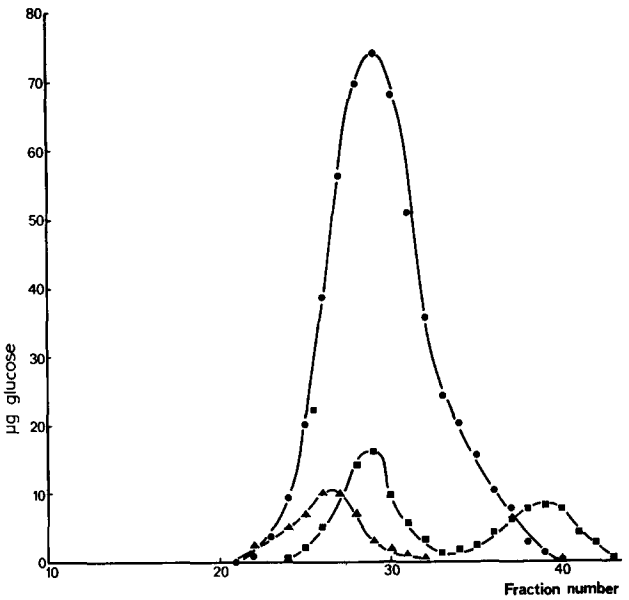


Fig. 1. Gel filtration of a crude enzyme preparation from *A. nidulans* on Sephadex G-100. One ml of the enzyme solution containing 1 mg protein was layered on a column (15 mm × 800 mm), equilibrated with 0.05 M citrate–phosphate buffer (pH 6.2) at 4°. α-1,3 glucan (●—●) soluble laminarin (■—■), and soluble starch (▲—▲) were incubated in the same buffer with 0.1 ml of the various 2-ml fractions for 1 h at 37°. Increase in reducing power was measured with neocuproine reagent and expressed as µg glucose.

RESULTS

Gel filtration on Sephadex G-100

The enzyme solution obtained after acetone precipitation was fractionated on a Sephadex G-100 column (15 × 800 mm). A typical elution pattern is shown in Fig. 1. It can be seen that there is only one peak for α-1,3 glucanase. In addition, there was some laminarinase and amylase activity in this preparation.

The pooled fractions (Numbers 26–32) contained 33 µg protein/ml and had a specific activity of 3.03 units/mg protein. The results of the inclusion of proteins with known molecular weight during gelfiltration on Sephadex G-100 suggested that α-1,3 glucanase had a molecular weight of about 36 000.

Michaelis–Menten kinetics

The influence of substrate concentration on the reaction velocity was determined. Substrates in concentrations ranging from 0.0625 to 4 mg/ml were incubated with 0.1 ml enzyme extract for 3 h at 37°. The results are expressed in a Lineweaver–Burke plot, as shown in Fig. 2. The K_m (g/ml) is $1.6 \cdot 10^{-3}$.

Action pattern

The action pattern of α-1,3 glucanase was investigated in two ways:

Firstly according to TUNG AND NORDIN[11], 0.9 ml α-1,3 glucan (2 mg/ml) was incubated with various amounts of enzyme. After appropriate dilution, activity was

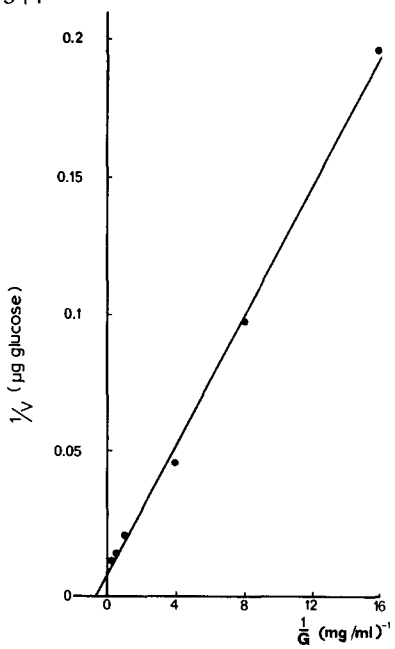


Fig. 2. Effect of substrate concentrations on reaction velocity (Lineweaver–Burke plot). Varying amounts of α-1,3 glucan were incubated with 0.1 ml enzyme (final volume of 1 ml). Incubation was carried out for 3 h at 37°. The reaction mixture was then assayed for increase in reducing power with the neocuproine reagent, expressed as μg glucose, and results plotted according to Lineweaver–Burke.

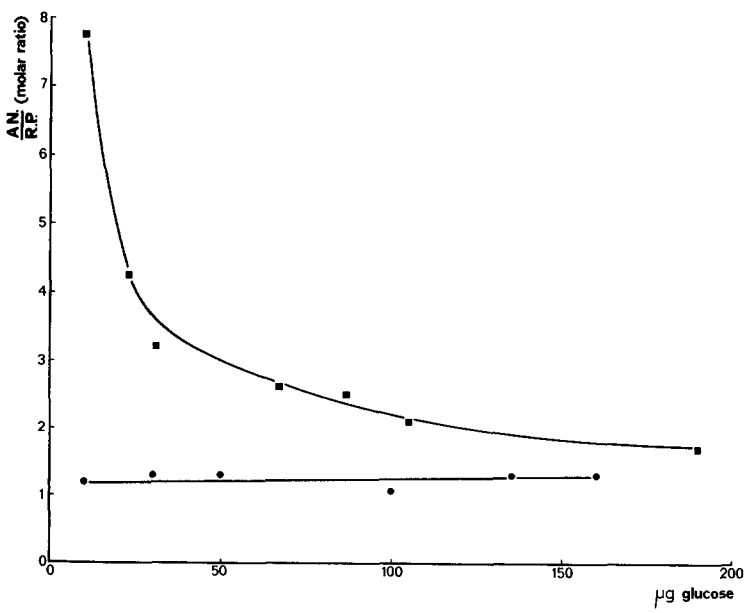


Fig. 3. Action pattern of the α-1,3 glucanase. α-1,3 glucan (●—●) was incubated with varying amounts of enzyme for 3 h at 37°. Insoluble starch (■—■) was incubated with varying amounts of α-amylase for 1 h at 37°. After filtrating off the undissolved substrate, the remaining solution was assayed for total carbohydrate with anthrone (AN) and for increase in reducing power (R.P.) with neocuproine. The ratio AN:R.P. is plotted against the glucose equivalents as measured with neocuproine.

measured as an increase in reducing power with the neocuproine reagent or as an increase in total dissolved carbohydrate with the anthrone reagent. The same method was used for a known endo-enzyme:α-amylase. As can be seen from Fig. 3, the results clearly indicate an exo-enzyme for α-1,3 glucanase and an endo-mechanism for α-amylase.

Secondly, a combined neocuproine–Glucostat determination was used; as can be seen from Fig. 4, a straight line with a slope of 45° can be drawn. This indicates that glucose accounts for all of the reducing power and is the only product of the enzyme, which is confirmed by the results of chromatography of the reaction product.

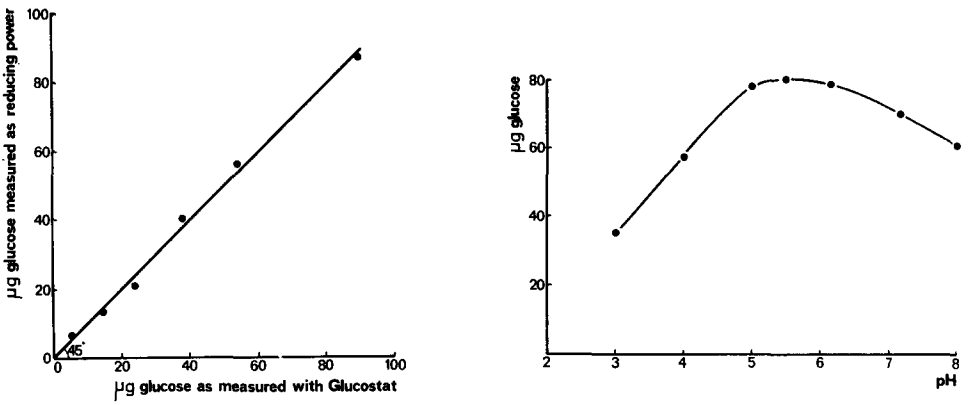


Fig. 4. Correlation between enzyme activity measured as increase in reducing power with neocuproine reagent and as glucose with the Glucostat reagent. α-1,3 glucan was incubated with varying amounts of enzyme for 3 h at 37°. After 5 min at 100°, one series was measured with neocuproine and expressed as μg glucose equivalents and the other series was made up to 2 ml (pH 7.) with 1 ml citrate–phosphate buffer (pH 8) and then assayed with the Glucostat reagent for 30 min at 37° and expressed as μg glucose.

Fig. 5. The pH optimum of α-1,3 glucanase. The activity was measured in citrate–phosphate buffer at various pH values. Reducing power was measured with neocuproine and expressed as μg glucose.

pH optimum

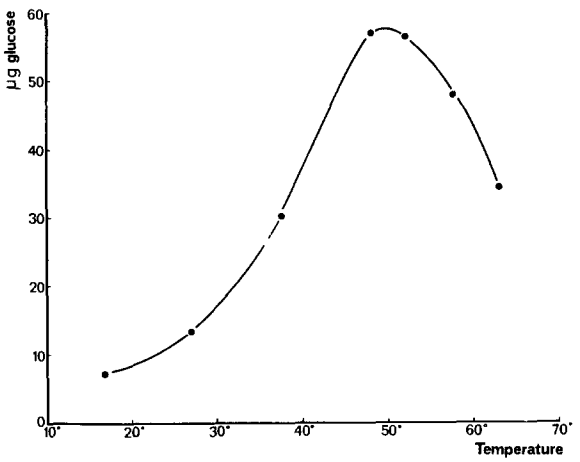
α-1,3 glucan was suspended in citrate–phosphate buffers with a pH ranging from 3.6 to 8, and incubated with an appropriate amount of enzyme. As can be seen from Fig. 5, α-1,3 glucanase is active over a wide range. Especially remarkable is the high activity at pH 8.

Temperature optimum

The assay mixture was first temperature-equilibrated, prior to the addition of enzyme. After incubation of the assay mixture for 1 h, the activity was measured with the neocuproine reagent and expressed as μg glucose. Fig. 6 shows that under these circumstances the optimum lies at 50°.

Heat inactivation

From 3 ml enzyme preparation incubated in a waterbath at 63°, 0.4 ml was removed after various intervals for the addition of 0.1 ml each to α-1,3 glucan, soluble



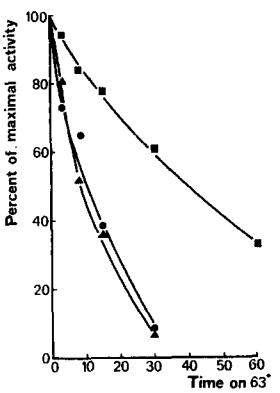


Fig. 6. Temperature optimum curve of α-1,3 glucanase. The assay mixture was equilibrated at the specified temperature prior to the addition of enzyme. Activity was measured with neocuproine after 1 h at the various temperatures and expressed as µg glucose.

Fig. 7. Heat inactivation curve at 63°. 3 ml enzyme preparation was incubated in a waterbath at 63°. At various times 0.4 ml was removed and of this 0.1 ml was added to α-1,3 glucan (●—●), soluble laminarin (■—■) and soluble starch (▲—▲), these mixtures were incubated for 3 h at 37°. Activity was measured as increase in reducing power with the neocuproine reagent and expressed as a percentage of maximum activity at 0 min at 63°.

laminarin, and soluble starch, after which these mixtures were incubated for 3 h at 37°. Activity was measured with the neocuproine reagent and expressed as a percentage of maximum activity at 0 min at 63°. As can be seen from Fig. 7 enzymes active toward laminarin are more stable than those active toward α-1,3 glucan and starch.

Complete degradation of α-1,3 glucan

10 mg of α-1,3 glucan from *A. nidulans*, 10 mg of α-1,3 glucan from *Schizophyllum commune* (prepared as described for α-1,3 glucan of *A. nidulans*, see under MATERIALS AND METHODS), and 10 mg of nigeran were incubated with 0.5 ml crude enzyme extract and 9.5 ml buffer for 24 h at 37°. α-1,3 glucan of *A. nidulans* underwent 90–98% degradation but α-1,3 glucan of *S. commune* only 45–52%; with nigeran, no activity was found.

Inhibition

To 1 mg of α-1,3 glucan, laminarin, or soluble starch, suspended or dissolved in 0.5 ml water, 0.1 ml enzyme extract was added as well as various amounts of $CuSO_4$ (final concentration : $2 \cdot 10^{-7}$ to $6.8 \cdot 10^{-2}$ M).

Activity was measured after 3 h incubation at 37°. The degradation of laminarin and starch was completely inhibited at $CuSO_4$ concentrations higher than $0.4 \cdot 10^{-2}$ M. The activity on α-1,3 glucan was not influenced by $CuSO_4$. Incubation with $ZnCl_2$ or $MgCl_2$ (final concentration $1.7 \cdot 10^{-2}$ M) did not inhibit the activity of α-1,3 glucanase.

Incubation with different amounts of glucose (from 0 to 1000 µg/ml) did not influence the activity of α-1,3 glucanase. The same holds for 10^{-8} to 10^{-2} mM EDTA.

DISCUSSION

Fungal α- and β-glucanases are generally extra-cellular enzymes with many properties in common. Temperature range, pH optimum, and the ease with which the enzyme α-1,3 glucanase can be isolated, are in agreement with this.

Unlike most glucanases, however, the α-1,3 glucanase is quite active at pH 8. This could be connected with the fact that at the time of maximum α-1,3 glucanase activity, the pH of the growth medium is about 8. The apparent K_m (g/ml) value ($1.6 \cdot 10^{-3}$) is rather high, but it should be kept in mind that α-1,3 glucan is an insoluble substrate.

As can be seen in Fig. 1, the enzyme preparation was not free of activity toward laminarin and starch, but after incubation with Cu^{2+} these two activities were abolished completely with no effect on α-1,3 glucanase activity. Furthermore, incubation of α-1,3 glucan with α-amylase, β-amylase, glucamylase, or all of these three enzymes combined, did not result in any degradation of α-1,3 glucan. It was found that the activity toward laminarin was much more heat-stable than that toward α-1,3 glucan. All this taken together, it seems justified to conclude that α-1,3 glucanase is specific for α-1,3 glucan and distinct from the laminarinases or amylases.

In a preliminary note on an α-1,3 glucanase, HASEGAWA *et al.*[12] suggested that the enzyme would appear an exo-enzyme. Subsequently, however, they published an extensive paper on an endo-α-1,3 glucanase[5] isolated in the same way from the same organism. In that paper they mentioned that an α-1,3 glucanase had been found earlier but not the fact that it appeared to be an exo-enzyme. This seems to imply that on further investigation they found, that the α-1,3 glucanase was of the endo-type.

The functional role of the α-1,3 glucanase in *A. nidulans* is not yet clear. But our experimental results (to be published) suggest that α-1,3 glucanase and its substrate α-1,3 glucan may play a role in morphogenesis. In addition, this enzyme can play an important role in determining the fine structure of polymers with α-1,3 linkages.

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