Carbohydrate Research, 144 (1985) 277–287
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

INVESTIGATIONS OF AN INDUSTRIAL β-D-GLUCANASE FROM *Trichoderma harzianum*

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(Received November 6th, 1984; accepted for publication in revised form, January 28th, 1985)

ABSTRACT

A commercial enzyme preparation of fungal origin *(Trichoderma harzianum)* contains a mixture of several β-D-glucanases. Ion-exchange chromatography, gel filtration, and chromatofocalisation permits separation of the crude enzyme preparation into the following enzyme fractions: two exo-(1→3)-β-D-glucanases, an endo-(1→6)-β-D-glucanase, and a β-D-glucosidase. The major fraction is an exo-(1→3)-β-D-glucanase having an isoelectric point of 7.8 and a molecular weight of ~40,000. This enzyme is able to digest the (1→3),(1→6)-β-D-glucans of the sclerotan type, such as cinerean.

INTRODUCTION

Soil-borne fungi of the genus *Trichoderma* have been especially studied for their cellulase activity[1,2]. Some preparations are now commercially available for use in the brewing industry; they are able to hydrolyse (1→3),(1→4)-β-D-glucans from barley[3]. An endo-(1→3)-β-D-glucanase has also been obtained by culture of *Trichoderma harzianum*[4].

Only a few studies have been reported on the (1→3)-β-D-glucanases from *Trichoderma*. Reese and Mandels[5], and Hadibi *et al.*[6], found a low laminaranase activity of the endo type in the culture filtrates of *Trichoderma* QM6a. However, Chesters and Bull[7] obtained strong exo-(1→3)-β-D-glucanase activities from another strain of *Trichoderma viride*. More recently, Bamforth[8] reported different specific and non-specific (1→3)-β-D-glucanases produced by *Trichoderma*.

The present paper describes the purification and characterization of the β-D-glucanases found in a new enzyme preparation (NOVOZYM SP-116**) from *Trichoderman harzianum* (Novo Industri, A/S, Bagsvaerd, Denmark). This preparation is proposed for use in wine making to hydrolyse cinerean, a (1→3),(1→6)-β-D-

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**Commercial name Glucanex.

0008-6215/85/$ 03.30

glucan of the sclerotan type, produced by the grape mold *Botrytis cinerea*, and responsible for clarification difficulties in wine technology[9–11].

EXPERIMENTAL

Materials. — The enzyme preparation was supplied as a brown powder. *O*-(Carboxyemethyl)cellulose was a gift from Institut du Pin, Université de Bordeaux I, France. Laminaran was obtained from Senn Chemicals, Dielsdorf, Switzerland. Modified laminaran (oxidised and reduced) and *Botrytis cinerea* (1→3),(1→6)-β-D-glucan (cinerean) were prepared according to procedures previously described[12]. Mutan, an insoluble (1→3)-α-D-glucan from *Streptococcus mutans,* was kindly donated by the Swiss Ferment Company Ltd., Basel. Rice starch was obtained from Prolabo, Paris, France, apple pectin from Sigma (St. Louis, Missouri, U.S.A.), and 4-nitrophenyl β-D-glucopyranoside from Fluka (Buchs, Switzerland). Pustulan, the (1→6)-β-D-glucan from the lichen *Umbilicaria pustulata*, was purchased from Calbiochem, La Jolla, CA, U.S.A.

All ion-exchange, gel-chromatography, and chromatofocalisation media were obtained from Pharmacia, Uppsala, Sweden. Elution gradients were optimized by an Ultrograd L.K.B. apparatus (Broma, Sweden).

General methods. — Reducing sugars were estimated by the Nelson–Somogyi method[13]. D-Glucose was specifically determined by an enzymic method[14]. Proteins were estimated by the Lowry method[15] or by column chromatography monitored by measuring the absorbance at 280 nm.

Assay of enzyme activities. — Polysaccharase activity was determined by incubating the enzyme (0.5 mL at a suitable dilution) with 0.5 mL of a solution or suspension containing 0.25% substrate in 10mM sodium acetate buffer, pH 5, for 10 min at 35°. The enzyme reaction was stopped by addition of 1 mL of the Somogyi copper reagent, and then the reducing power of the digest was measured. One unit of enzyme activity is defined as the amount of enzyme that forms in 10 min an increase of reducing power equivalent to 1 μmol of glucose.

β-D-Glucosidase activity was measured according to the method of Tingle and Halvorson[16] using *p*-nitrophenyl β-D-glucopyranoside (NP-Glc). The enzyme solution was added to 2.9 mL of a mixture containing 2.5 mL of 6.7mM potassium phosphate buffer (pH 6.8), 0.3 mL 10mM NP-Glc, and 0.1 mL of reduced glutathione. Absorbance was monitored at 400 nm. One unit of enzyme activity is defined as the amount of enzyme necessary to bring about an absorbance change of 0.001 per min per 3 mL of mixture.

Chromatography on DEAE-Sepharose. — The crude enzyme preparation (100 mg) was dissolved in 5 mL of water and the solution was desalted by filtration through a column (1.6 × 60 cm) of Sephadex G25 eluted with water.

The desalted sample (45 mL) was applied to a column (1.6 × 14 cm) DEAE-Sepharose initially washed with water. An optimized sodium chloride gradient (0→1M) was then applied. Fractions of 3 mL were collected and assayed for β-D-

glucosidase, (1→3)-β-D-glucanase, and (1→6)-β-D-glucanase activities. Peaks denoted G_1, G_2, and G_3 were collected. The purification steps of these fractions involved gel filtration, chromatography on CM-Sepharose and Sephacryl S200, and chromatofocalisation.

Gel filtration on Sephacryl S200. — The column (1.6 × 76 cm) was eluted with a 10mM sodium acetate buffer.

Chromatography on CM-Sepharose. — The column (1.6 × 14 cm) was first equilibrated with sodium acetate buffer 10mM (pH 6.5) and then submitted to a 0→1M gradient of NaCl.

Chromatofocalisation. — This analysis was performed on a column (0.9 × 30 cm) packed with the poly-buffer-exchanger named P.B.E. 9–4. This gel was first equilibrated at pH 8.3 with starting buffer (25mM Tris-HCl). The desalted enzyme sample was dissolved in this eluent (5 mL) before being applied to the column. Fractions of 3 mL were collected and assayed for proteins, pH, and (1→3)-β-D-glucanase activity.

Action patterns of β-D-glucanases. — The action patterns of the isolated β-D-glucanases were determined on laminaran, pustulan, and cinerean according to the method of Tung and Nordin[17] or by the comparing activities against laminaran in one instance, and against periodate-oxidized and sodium borohydride-reduced laminaran in the other.

Activities in presence of inhibitors. — The effect of metal ions on the exo-(1→3)-β-D-glucanase activity was examined in the range of 5 to 0.05mM. The effect of phosphate ions was also assayed between 50 and 5mM. The effect of ethylenedinitrilo(tetraacetic acid), CN^-, and D-glucono-1,4-lactone was also assayed. Relative activity was expressed as percentage of enzyme activity in the absence of inhibitors.

K_m *values.* — Michaelis constants were obtained according to Lineweaver and Burk[18] by measuring initial rate of hydrolysis at various concentrations of the substrate (laminaran and cinerean).

RESULTS AND DISCUSSION

Carbohydrase activities in the crude enzyme preparation. — (1→3)-β-D-Glucanase (laminaranase) and (1→6)-β-D-glucanase (pustulanase) represent the main activities of the crude preparation (Table I). This material is also able to digest a (1→3),(1→6)-β-D-glucan such as cinerean, but more slowly than laminaran. Amylase, D-galacturonanase, and (1→3)-α-D-glucanase activities were also detected.

Fractionation of crude enzyme preparation and action patterns of the separated enzymes. — Fig. 1 summarizes the fractionation of Novozym SP-116.

(a) Chromatography on DEAE-Sepharose. By chromatography on DEAE-Sepharose (Fig. 2), the desalted sample gives 3 peaks of β-D-glucanase activity: a

TABLE I

CARBOHYDRATE ACTIVITIES IN THE CRUDE PREPARATION NOVOZYM 116

Substrate	*Glucosidic linkage*	*Activity (Unit/mg)*	*Relative activity*
Laminaran	β-(1→3)	45.0	100
Pustulan	β-(1→6)	11.5	24
Starch	α-(1→4)	0.5	14
Cellulose	β-(1→4)	0.0	0
Dextran	α-(1→6)	0.0	0
Pectin		3.3	8
Mutan	α-(1→3)	1.6	5
Cinerean	β-(1→3) β-(1→6)	9.0	20

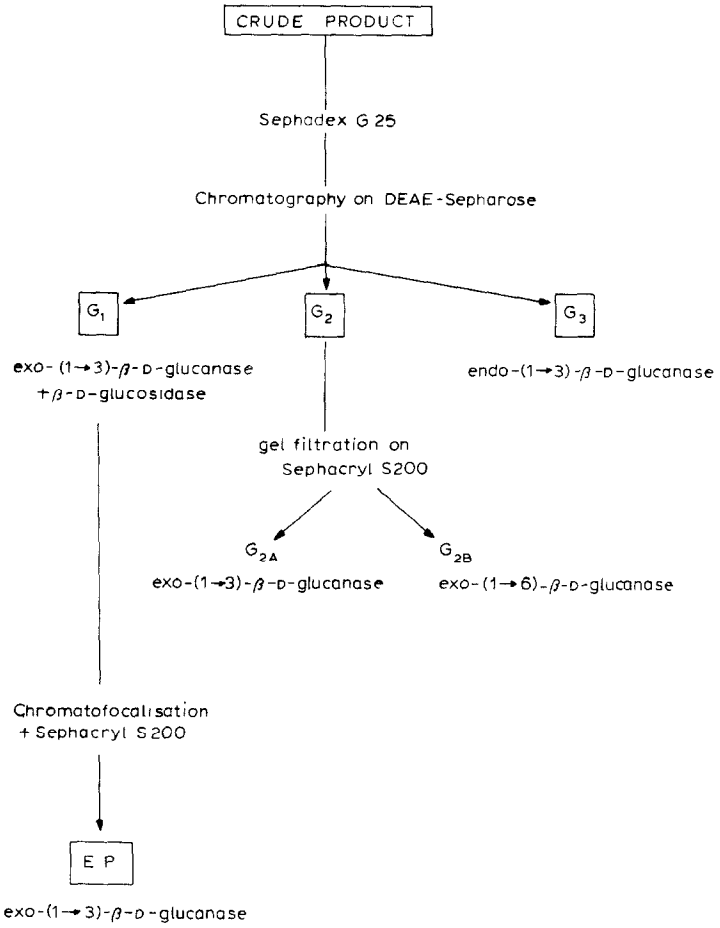

Fig. 1 Purification of the β-D-glucanase preparation

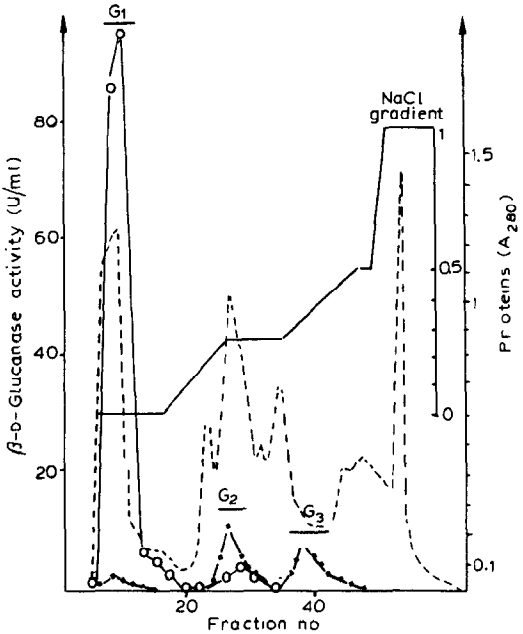

Fig. 2. DEAE-Sepharose chromatography of the desalted sample (column: 1.6 × 14 cm). Elution water then NaCl gradient (0–1M); ----: A_{280} (protein); O—O: (1→3)-β-D-glucanase activity ◆—◆; (1→6)-β-D-glucanase activity.

major fraction (G_1) eluted with the sodium chloride gradient and two minor fractions (G_2) and (G_3) also eluted with the sodium chloride gradient.

G_1 hydrolyses mainly laminaran and has a very poor (1→6)-β-D-glucanase activity. This fraction is also able to digest NP-Glc and gentiobiose (β-D-glucosidase activity). G_2 has (1→3)- and (1→6)-β-D-glucanase activity, whereas G_3 possesses only (1→3)-β-D-glucanase activity.

Surprisingly, the cinerean is hydrolysed only by G_1, even though G_2 contains the two activities [(1→3) and (1→6)-β-D-glucanase] necessary for cleaving the glycosidic linkages.

(b) Further purification of G_1 and G_2. The β-D-glucosidase activity of G_1 may be partially separated from (1→3)-β-D-glucanase activity by CM-cellulose chromatography (Fig. 3).

Gel filtration of G_2 on Sephacryl S_{200} allows the separation of two subfractions: a (1→3)-β-D-glucanase (G_{2A}) and a (1→6)-β-D-glucanase (G_{2B}) (Fig. 4).

(c) Action patterns of G_1, G_{2A}, G_{2B}, and G_3. The action patterns of the β-D-glucanases obtained have been studied. The ratio between the increase of the reducing power (RP) of the incubated solution and the amount of glucose (Glc) liberated has been used to determined the type of hydrolysis mechanism involved. Fig. 5 shows that G_1 and G_{2A} hydrolyse laminaran in an exo manner, but that G_3 is an endo-(1→3)-β-D-glucanase. The action pattern of G_{2B} on pustulan is exo.

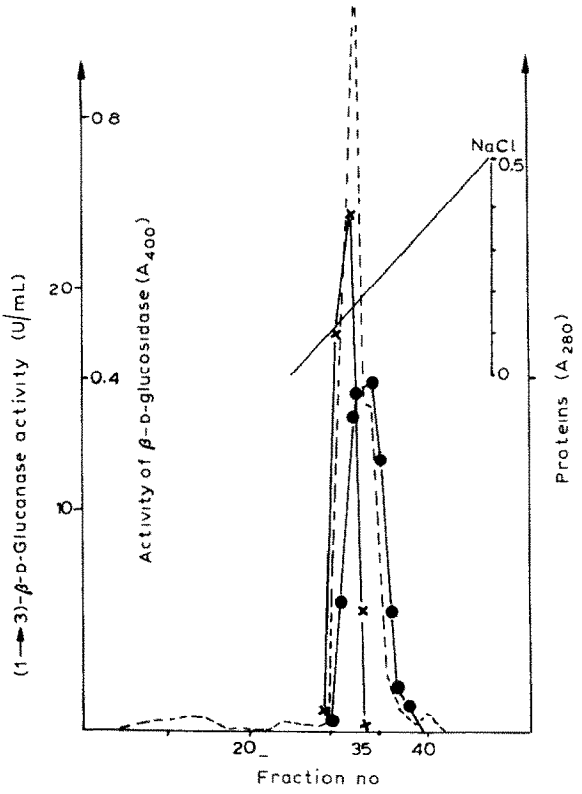

Fig. 3. Partial separation of β-D-glucosidase and exo-(1→3)-β-D-glucanase by CM-Sepharose chromatography. Column 1.6 × 14 cm; Elution: water then NaCl gradient (0–0.5M); ×—×: (1→3)-β-D-glucanase activity; ●—●: β-D-glucosidase activity; ----: A_{280} (protein).

These results were confirmed by measuring the activity of the different fractions against native and modified laminarans (Table II). G_1 and G_{2A} degrade reduced laminaran; it is thus demonstrated that these enzymes degrade linear β-D-glucan in a stepwise manner, beginning from the nonreducing end. However, G_3 hydrolysis oxidized and reduced laminaran as well as native laminaran in a random fashion; this endo-activity liberates oligosaccharides.

(d) Further characterization of G_1. The freeze-dried fraction G_1 was further purified by chromatofocalisation (see Experimental section). Exo-(1→3)-β-D-glucanase activity was detected in a single and symmetrical peak of protein eluted from the column when the pH of eluent was about 7.8. This value is near the isoelectric point of the enzyme (Fig. 6). The last purification step of exo-(1→3)-β-D-glucanase involved gel filtration on a Sephacryl S_{200} column calibrated with proteins of known molecular weights. The molecular weight of the enzyme is about 40 000 daltons. A 7-fold purification of the exo-(1→3)-β-D-glucanase was obtained after five purification steps (Table III).

General properties of the exo-(1→3)-β-D-glucanase. — The effects of pH,

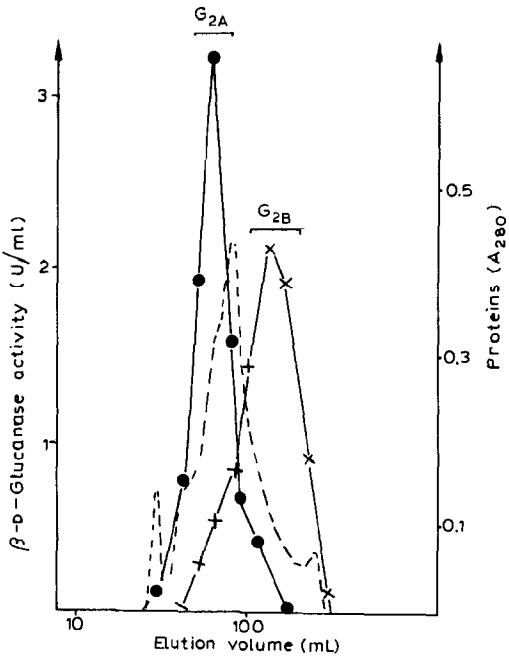

Fig. 4. Gel filtration of G_2 on Sephacryl S200; Column 1.6 × 76 cm; ----: A_{280} (protein); ●—●: (1→3)-β-D-glucanase activity; ×—×: (1→6)-β-D-glucanase activity.

temperature, SO_2, and ethanol on the activity and the stability of the exo-(1→3)-β-D-glucanase have already been published in a previous paper[9].

(a) Effect of inhibitors (Table IV). The activity of the enzyme was not affected by Mg^{2+} and Pb^{2+}, was moderately inhibited by Mn^{2+}, Fe^{2+}, and Cu^{2+}, and was strongly influenced by Hg^{2+} ions. CN^- (10^{-1} or 10^{-2}M) has no effect. $PO4^{3-}$ is an

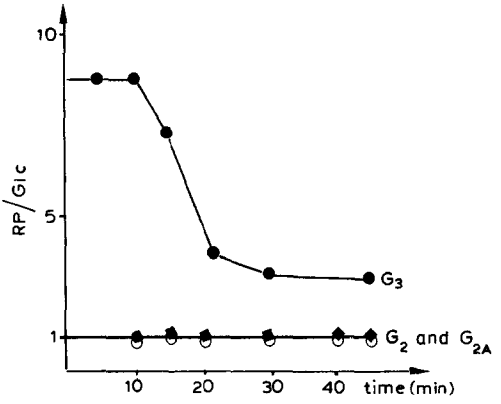

Fig. 5. Action patterns of β-D-glucanases found in G_1, G_{2A} and G_3; changes in RP/Glc ratio during Laminaran hydrolysis by G_1: ○—○, G_{2A}: ◆—◆, G_3: ●—●; (RP, reducing power: Glc, glucose).

TABLE II

HYDROLYSIS OF NATIVE LAMINARAN (A), REDUCED LAMINARAN (B), AND OXIDIZED LAMINARAN (C), BY THE 3 GLUCANASE FRACTIONS ISOLATED FROM THE ENZYME PREPARATION (G_1, G_{2A}, G_3)

Substrate (0.5 mL)	*Enzymatic fraction (nature and volume)*		*Increase of reducing power (μg/glucose/mL)*
A	G_1	20 μL	42
B	G_1	20 μL	36
C	G_1	20 μL	0
A	G_{2A}	200 μL	102
B	G_{2A}	200 μL	88
C	G_{2A}	200 μL	0
A	G_3	200 μL	139
B	G_3	200 μL	100
C	G_3	200 μL	137

inhibitor at 10^{-1}M (Table V). A similar effect of phosphate ions on β-D-glucanases has been already reported[19].

D-Glucono-1,4-lactone is known to be a competitive inhibitor of β-D-glucosidases and nonspecific exo-β-D-glucanases, and to have poor inhibitory effect on specific exo-(1→3)-β-D-glucanases[20,21]. This is verified by our results.

(b) Michaelis constants. The K_m value for the enzyme when incubated against laminaran was 0.015 mg/mL, and 0.2 mg/mL when tested on cinerean (Table VI). These values are the same as those given by Nelson *et al.*[22], for the K_m value of

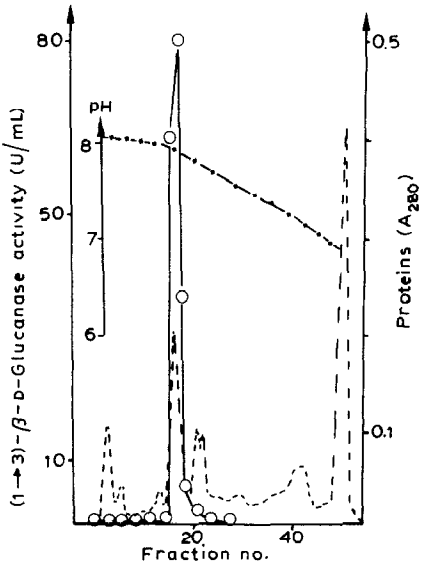

Fig. 6. Chromatofocusing of G_1. ○—○: (1→3)-β-D-glucanase activity (U/mL); ----: A_{280} (protein); ■—■: pH.

TABLE III

PURIFICATION OF EXO-β-(1→3)-D-GLUCANASE FROM THE *Trichoderma* INDUSTRIAL ENZYME PREPARATION

Purification step	*Volume (mL)*	*Total activity (U/mL) on*		*Protein (mg)*	*Specific activity (U/mg protein) on*		*Yield (%)*
		Laminaran	*Pustulan*		*Laminaran*	*Pustulan*	
Crude extract (100 mg)	5	4500	1150	83	54	14	100
Sephadex G-25	20	4100	[a]	60	[a]	[a]	90
DEAE-Sepharose							
peak 1 G_1	51	3115	58	24	130	2	69
peak 2 G_2	38	110	188	13	8	14	2
peak 3 G_3	52	202	0	10	20	0	4
Chromatofocussing	15	882	0	[a]	[a]	[a]	20
Sephacryl S200	20	470	0	1.3	362	0	10

[a]Undetermined.

TABLE IV

INFLUENCE OF METAL IONS ON THE EXO-(1→3)-β-D-GLUCANASE (FRACTION G_1); RELATIVE ACTIVITY (%)

Cation control (100)	*5mM*	*0.5mM*	*0.05mM*
Mn^{2+} ($MnCl_2$)	48	60	87
Ca^{2+} ($CaCl_2$)	109	120	99
Mg^{2+} ($MgCl_2$)	100	91	97
Mg^{2+} ($MgSO_4$)	79	95	102
Ba^{2+} ($BaCl_2$)	128	107	86
Hg^{2+} ($HgCl_2$)	0	0	60
Fe^{2+} ($FeSO_4$)	53	69	80
Cu^{2+} ($CuSO_4$)	41	64	99
Pb^{2+} (acetate)	94	98	99

TABLE V

ACTIVITY OF FRACTION G_1 (%) IN THE PRESENCE OF PHOSPHATE IONS, OR D-GLUCONO-1,4-LACTONE

Control (Sodium acetate)	*50mM*	*5mM*
	100	100
KH_2PO_4	38	97
Na_2HPO_4	0	113
$(NH_4)_2HPO_4$	0	100
D-Glucono-1,4-lactone	80	85

TABLE VI

MICHAELIS CONSTANTS OF THE EXO-β-(1→3)-D-GLUCANASE OF *Trichoderma harzianum*

Substrate	V_m (μmol/min)[a]	K_m (mg/mL)
Cinerean	0.12	0.2
Laminaran	0.3	0.015

[a]For 3 U of laminaranase activity per mL of mixture.

Basidiomycete QM 806 exo-(1→3)-β-D-glucanase. The K_m values for many other (1→3)-β-D-glucanases are higher[12].

CONCLUSION

The present studies have demonstrated the presence of several β-D-glucanases in a commercially available enzyme preparation (Novozym SP-116): two exo-(1→3)-β-D-glucanases, one endo-(1→3)-β-D-glucanase, one exo-(1→6)-β-D-glucanase, and a β-D-glucosidase. These enzymes may be readily purified and should provide useful tools for the structural analysis of β-D-glucans.

The fraction eluted with water from DEAE-Sepharose contains the two enzymes of technological interest for hydrolysis of cinerean: the major exo-(1→3)-β-D-glucanase and the β-D-glucosidase. The first enzyme, like the exo-(1→3)-β-D-glucanases already known, can bypass (1→6)-β-D-linkages liberating glucose and gentiobiose, which is further cleaved in glucose by the β-D-glucosidase activity.

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