



PURIFICATION OF AN ARBUSCULAR MYCORRHIZAL ENDOGLUCANASE FROM ONION ROOTS COLONIZED BY *GLOMUS MOSSEAE*

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Summary—An arbuscular endoglucanase (EC 3.2.1.4) was purified to homogeneity from roots of onion (*Allium cepa* cv. Babosa) colonized by the arbuscular mycorrhizal fungus *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe. The stepwise purification procedure consisted of Filtron concentration (10 kDa), anion-exchange chromatography, anion-exchange fast protein liquid chromatography, and electroelution from polyacrylamide gels. Pure endoglucanase had a specific activity of 2500 units mg⁻¹ protein, and was purified 198-fold with a yield of 0.6 µg enzyme g⁻¹ root. The endoglucanase has a relative molecular weight of about 27 kDa, and behaves as a monomer in its native form. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

The colonization of plant roots by arbuscular mycorrhizas (AM) fungi involves the formation of intercellular hyphae and intracellular arbuscules in the root (Bonfante-Fasolo, 1984). Electron microscopic observations suggest that the establishment of an intracellular symbiosis between AM fungi and plant roots requires penetration of the host cell by fungus. Cell wall-hydrolyzing enzymes such as cellulases must be involved in this process (García-Romera *et al.*, 1990). Considerable attention has been devoted to the study of the role of cellulase enzymes in physiological and pathological changes in plants (Mendgen and Deising, 1993; Beguin and Aubert, 1994), but research on these enzymes in plant roots, and information on their mode of action in the process of penetration and development of symbiotic microorganisms, is scarce (Chalifour and Benhamou, 1989). The presence of endoglucanase activity, which belongs to the cellulase system (Coughlan and Ljungdhal, 1988), in extracts of external mycelia of *G. fasciculatum* and in *G. mosseae* spores indicates that AM fungi produce this enzyme (García-Garrido *et al.*, 1992a,b). Similar observation have been made with other mutualistic microorganisms (Mateos *et al.*, 1992). Moreover, endoglucanase activity was higher in AM-colonized plants than in non-AM colonized plants (García-Garrido *et al.*, 1992c). Maximum endoglucanase activity coincided with the beginning

of entry point formation and arbuscule development (García-Garrido *et al.*, 1992b). The formation of entry points and arbuscules requires that fungal hyphae pass through the cell wall (Bonfante-Fasolo, 1984). These observations suggest that the fungus produces endoglucanase enzymes which allow penetration of the cell wall. However, it is difficult to establish a direct relationship between endoglucanase activity and the development of the different fungal structures. Some of the endoglucanase activities found in AM colonized roots can be attributed to the AM fungus, since the enzyme activity found in external mycelia and in mycorrhizal root extracts showed the same electrophoretic mobility (García-Garrido *et al.*, 1992b).

To date, no AM endoglucanase has been purified. The purification of AM endoglucanase is the first step toward obtaining monospecific antibodies against the pure protein, which could be used in comparative immunological studies of plant endoglucanases, and would make immunological studies of the development of the fungus inside the root possible. Our aim was to purify from AM-colonized plants the endoglucanase protein which showed the same electrophoretic mobility as the external mycelium of *G. mosseae*.

MATERIALS AND METHODS

Biological material and growth conditions

Plants were grown in 300 ml capacity open pots of soil collected from the Province of Granada, Spain. The soil was a calcixerollic xerochrept type,

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pH 7.6 (García-Romera and Ocampo, 1988). It was steam-sterilized and mixed with sterilized quartz sand in a ratio of 1:1 (V:V). Onion (*Allium cepa* cv. Babosa) was used as the test plant. Seeds were sown in moistened sand, and after 2 weeks seedlings were transplanted to the pots and grown under greenhouse conditions. Natural light was supplemented by Sylvania incandescent and cool-white lamps, 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 400–700 nm; with a 16–8 h light–dark cycle at 25–19°C and 50% relative humidity. Plants were watered from below using a capillary system, and fed with a nutrient solution (Hewitt, 1952), lacking phosphate for AM-inoculated plants.

The AM inoculum consisted of 5 g of rhizosphere soil from alfalfa plant pot culture of an isolate of *G. mosseae* (Nicol. and Gerd.) Gerd. and Trappe which contained spores (10 sporocarps g^{-1} with 1 to 5 spores per sporocarp), mycelium and colonized root fragments. Uninoculated plants were given filtered leachings from the inoculum soil. Soil filtrate (Whatman no.1 filter paper) from the rhizosphere of mycorrhizal plants was added to the AM uninoculated treatment. The filtrate contained common soil microorganisms, but no propagules of AM.

External mycelia were isolated from roots of 35-day-old onion colonized with *G. mosseae*. The roots were washed and rinsed gently with sterilized water, and the external mycelia collected with forceps under a dissecting microscope.

Mycorrhizal measurements

Plants were harvested after 35 d. The root system was washed and rinsed three times with sterilized distilled water and parts of the root system (2 g f.w.) were cleared and stained (Phillips and Hayman, 1970). The percentage of total root length which was colonized with arbuscular mycorrhiza was measured by the gridline intersect method (Giovannetti and Mosse, 1980).

Preparation of extracts for enzyme assays and purification of enzyme

Roots (150 g of AM plants and 16 g of non-AM plants, f.w.) were frozen in liquid N_2 and pulverized in a mortar. The resulting powder was homogenized in 0.1 M Tris-HCl buffer (pH 7.0) containing 12% polyvinyl-pyrrolidone (PVPP), 10 mM MgCl_2 , 10 mM NaHCO_3 , 10 mM β -mercaptoethanol, 150 μM phenylmethyl sulfonyl fluoride (PMSF) and 0.3% (W/V) Triton X-100 (Buffer A) in a ratio of 1:3 (W:V). Sodium azide (0.03%) was also added to this solution. The homogenate was filtered through several layers of cheesecloth, centrifuged at 20,000g for 15 min, and the pellet resuspended and washed by centrifugation with the same buffer three times. The final supernatant was concentrated with an Omega membrane (10 kDa, Filtron Tech. Co.) and dialyzed overnight against several hundred volumes of diluted (1:9; V:V) buffer A. The dialyzed solution

was clarified by centrifugation at 25,000g for 20 min and used as a crude enzyme extract. The enzyme solution was applied to a DEAE-Sephadex A-50 column (2.6 \times 40 cm) equilibrated with buffer A. Endoglucanase activity was eluted with 500 ml of a linear salt gradient from 0 to 1 M NaCl in buffer A. Endoglucanase activity of these fractions was analyzed by PAGE, using gels containing carboxy methyl cellulose (CMC). The active fractions were desalted with PD 10 columns (Pharmacia) and concentrated to 5 ml by ultrafiltration with a PM-10 membrane. The concentrated DEAE-Sephadex fractions were applied to a Mono Q HR 5/5 column equilibrated with buffer A, using a fast protein liquid chromatography (FPLC) system equipped with an LCC-500 PLUS controller, two P-500 pumps, an MV-7 motor valve, and a UV-M monitor connected to a REC-482 two-channel recorder and a FRAC-200 fraction collector (Pharmacia LKB). Sample loops of 1-ml volume were used and all FPLC fractionations were done at room temperature. The column was washed with 25 ml of buffer A, and was then eluted with 32 ml of a linear salt gradient from 0 to 1 M NaCl in buffer A. Fractions of 1 ml were collected at a flow rate of 0.75 ml min^{-1} . Each activity peak was combined separately and concentrated by ultrafiltration to 2 ml and analyzed by native PAGE. The endoglucanase protein was electroeluted from PAGE gel slices showing endoglucanase activity by using a 422 Electro-eluter (Bio-Rad) according to the manufacturer's instructions.

External mycelia were frozen in liquid N_2 and finely pulverized in a mortar. The resulting powder was suspended (30 mg ml^{-1}) in the same extractant solution as for roots. The suspension was briefly sonicated (1 min 5 times at 80 W) and centrifuged at 20,000g for 15 min; then the pellet was resuspended and sonicated again, and was washed by centrifugation with the same buffer three times. The combined supernatants were concentrated by ultrafiltration with PM-10 membranes (AMICON Co.), and used as a crude enzyme extract.

Enzyme assays and protein determination

Endoglucanase activity (EC 3.2.1.4) was assayed by the viscosity reducing method with CMC as the substrate. Viscosity reduction was determined in a Cannon-Fenske viscosimeter (5354/2) at 37°C. The reaction mixture (6 ml) contained 5 ml 0.5% substrate in 50 mM citrate phosphate buffer (pH 5.0) and 1 ml of either root or fungus extracts. One unit of enzyme activity was expressed on a relative activity (RA) basis (reciprocal of time in min for 50% viscosity loss \times 1000) (García-Garrido *et al.*, 1992b).

Controls for all enzyme assays were autoclaved enzyme extracts and buffers, and 0.03% sodium azide was added to all reaction mixtures. Protein

was determined by the method of Bradford (1976) with bovine serum albumin as the standard.

Polyacrylamide gel electrophoresis

Non-denaturing linear gradient electrophoresis of endoglucanase enzymes was performed in polyacrylamide gels (4–12%) amended with 0.1% CMC in 50 mM Tris–0.1 M glycine buffer (pH 8.8) (García-Garrido *et al.*, 1992b).

Gels measuring 16 × 18 cm and 1.5 mm thick were prepared using a gradient gel casting chamber (LKB). The electrode tanks contained the same Tris-glycine buffer (pH 8.8) as used in the gel. After electrophoresis of the gel for 30 min, the wells were filled with 75 μ l of either root or fungus extracts and 1 μ l 0.05% (W:V) bromophenol blue. Electrophoresis was done at 4°C and at a constant current of 20 mA per gel for 5 h.

After electrophoresis the gels were kept immersed in 100 ml of 50 mM citrate-phosphate buffer (pH 5.0) at 37°C for 15 h, and were then stained with 0.1% Congo red for 30 min. This was followed by washing in 1 M NaCl until colourless bands became visible against a red background.

The native mol. wt of purified endoglucanase protein was determined by non-denaturing linear gradient (4–12%) PAGE plus 0.1% CMC. After

electrophoresis, the gel was divided into two parts, one was used to visualize endoglucanase activity as described above, and the other, with the standards and the endoglucanase proteins, was silver stained (Sigma). The standards used were: urease (trimer, Mr = 272,000), BSA (monomer, Mr = 66,000, dimer, Mr = 132,000), ovoalbumin (Mr = 45,000), α -lactoalbumin (monomer, Mr = 14,000, tetramer, Mr = 56,800). The subunit size was determined by SDS-PAGE after heating the proteins at 100°C for 5 min in the presence of 2% SDS and 5% 2-mercaptoethanol. Electrophoresis was done on linear gradient (4–12%) acrylamide-SDS gels as described by Laemmli (1970). Standards used were: phosphorylase b (Mr = 97,400), BSA (Mr = 66,000), ovoalbumin (Mr = 45,000), carbonic anhydrase (Mr = 31,000), soybean trypsin inhibitor (Mr = 21,500) and lysozyme (Mr = 14,400). Proteins were visualized by silver staining (Sigma).

RESULTS AND DISCUSSION

Important changes in plant metabolism are thought to occur after symbiosis with AM fungi has been established (Arines *et al.*, 1993; Palma *et al.*, 1993), although very little is known about the biochemical mechanisms responsible for this associ-

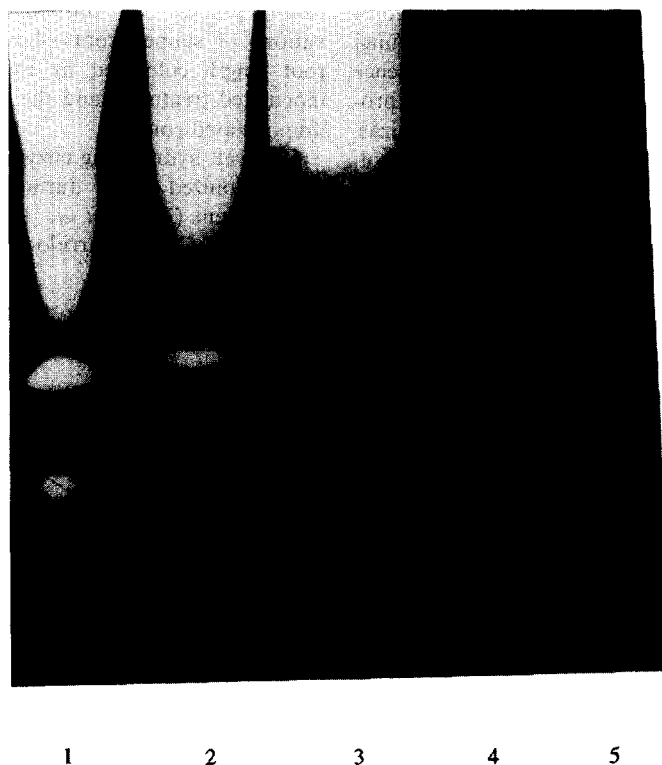


Fig. 1. Non-denaturing PAGE of cellulase. Lane 1; extracts from 35-day-old uncolonized onion roots; lane 2; extracts from 35-day-old AM-colonized onion roots; lane 3; extracts from external mycelium of *G. mosseae*; lane 4; endoglucanase activity after DEAE-Sephadex (Peak I₆); lane 5: endoglucanase activity after FPLC (Peak M). The gel was stained with Congo red and destained as described in Materials and Methods.

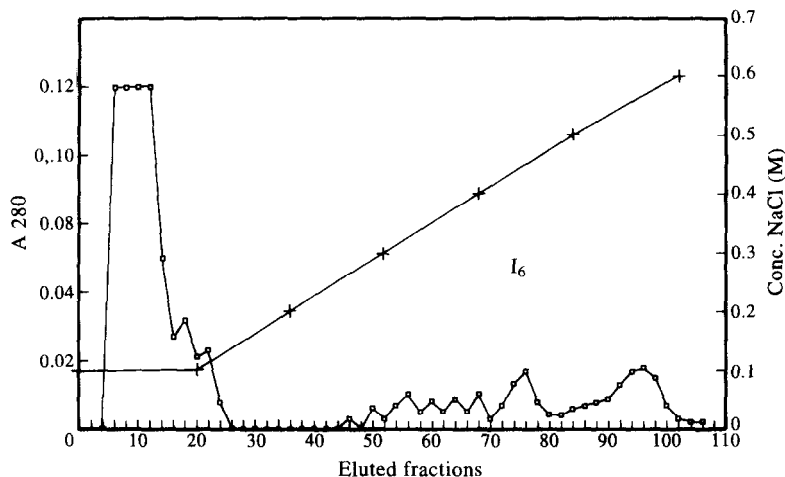


Fig. 2. Fractionation of crude enzyme extracts of AM onion roots on a DEAE-Sephadex column: ■ = protein concentration; + = M NaCl gradient profile. I_6 = Peak of endoglucanase protein.

ation. Increasing attention is being given to the study of the biochemical processes involved in the colonization of roots by the AM fungi (Bonfante-Fasolo *et al.*, 1990). In spite of the importance of endoglucanases in the penetration of many microorganisms through the plant cell wall, little is known of the role these enzymes may play in the colonization of plant roots by AM fungi (García-Garrido *et al.*, 1992b). However, since AM mycorrhizal fungi have not yet been cultured axenically in the absence of plant roots, it is difficult to confirm the production of cellulytic enzymes by AM mycorrhizas, or their possible role in the colonization of the root. Moreover, it is difficult to detect the activity of cell wall-degrading enzymes, since breakdown of the walls must be highly localized and is difficult to

detect during fungal development (García-Garrido *et al.*, 1992a). Previous studies indicated that endoglucanase activity was higher in mycorrhizal than in non-mycorrhizal onion roots at 35 days after inoculation with the fungus (García-Garrido *et al.*, 1992c). We therefore harvested the roots at that time to obtain maximum recovery in the purification process. At this time microscopic observations of stained roots showed $55 \pm 10\%$ of the root length colonized by the AM fungus in the inoculated treatment and the absence of fungi from uninoculated controls.

Cell-wall hydrolyzing enzymes are present in non-AM colonized roots during normal growth and development (Byrne *et al.*, 1975, García-Garrido *et al.*, 1992b). García-Garrido *et al.* (1992b), observed

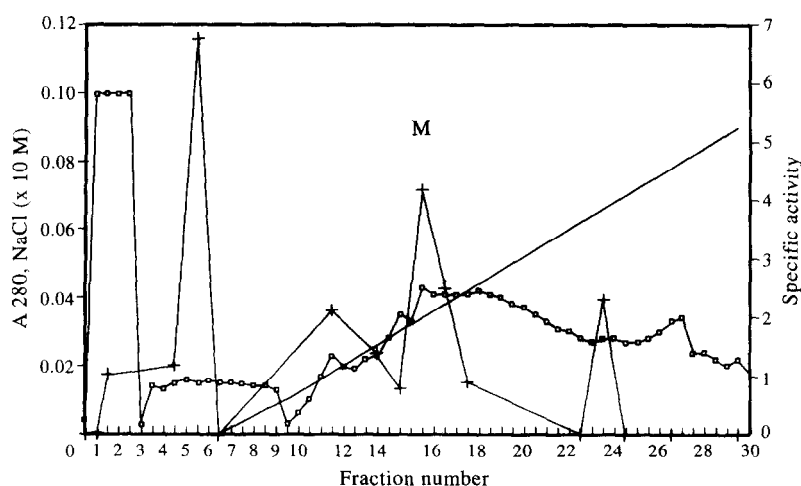


Fig. 3. Fractionation of concentrated DEAE-Sephadex fractions on a Mono Q column using a FPLC system: + = endoglucanase activity; ■ = protein concentration. The unbroken line represent the gradient profile (M NaCl). M = Peak of mycorrhizal endoglucanase protein.

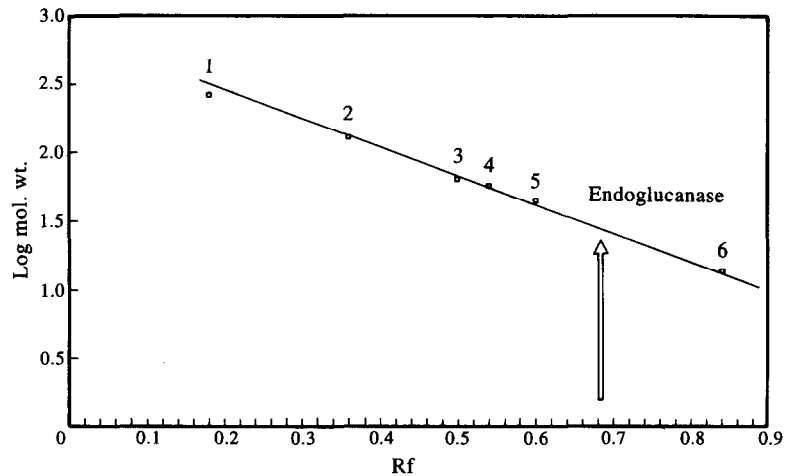


Fig. 4. Mol. wt estimation of AM endoglucanase by non-denaturing PAGE in gradient gels (4–12% acrylamide). The log mol. wt of standard protein markers was plotted against their Rf values. 1 = Urease; 2 = BSA (dimer); 3 = BSA (monomer); 4 = α -lactoalbumin; 5 = ovoalbumin; 6 = α -lactalbumin.

several bands of endoglucanase activity in 35-day-old onion plants (Fig. 1), and the electrophoretic pattern in crude enzyme extracts differed between mycorrhizal and non-mycorrhizal plants (Fig. 1, lanes 1 and 2). The presence of bands in AM mycorrhizal roots which were absent from non-mycorrhizal roots and from external mycelia suggests that some of this activity may be produced by the fungus inside the root, or may be induced by the fungus in the plant (García-Garrido *et al.*, 1992b). Some of this endoglucanase activity can be attributed to the extramatrix phase of the AM fungus, since at least one of the endoglucanase activities found in the mycorrhizal root and in the external mycelium extracts had the same electrophoretic mobility (Fig. 1, lanes 2 and 3, respectively).

In the early stages of purification of mycorrhizal endoglucanase, attempts were made to precipitate the enzyme with ammonium sulfate (García-Garrido *et al.*, 1992b). However, the yield with this method was low, and in addition, a creamy layer of fat produced turbidity in the solutions, which was difficult to remove. In contrast, concentration with Omega membranes (10 kDa, Filtron Tech. Co.) resulted in better recoveries of enzyme activity. The use of DEAE-Sephadex columns was useful to partially purify and concentrate the enzyme solution prior to the FPLC purification step, and increased the specific activity of endoglucanases by 2.1-fold. One of the peaks of endoglucanase activity eluted from the DEAE-Sephadex column (Peak I₆, Fig. 2), showed the same electrophoretic pattern as the external mycelium (Fig. 1, lanes 4 and 3).

FPLC on an anion-exchange Mono Q column separated four peaks of endoglucanase activity (Fig. 3), which were analyzed by native PAGE.

Peak M (Fig. 3), which bound to the Mono Q column, was characterized as a mycorrhizal endoglucanase because its electrophoretic mobility was similar

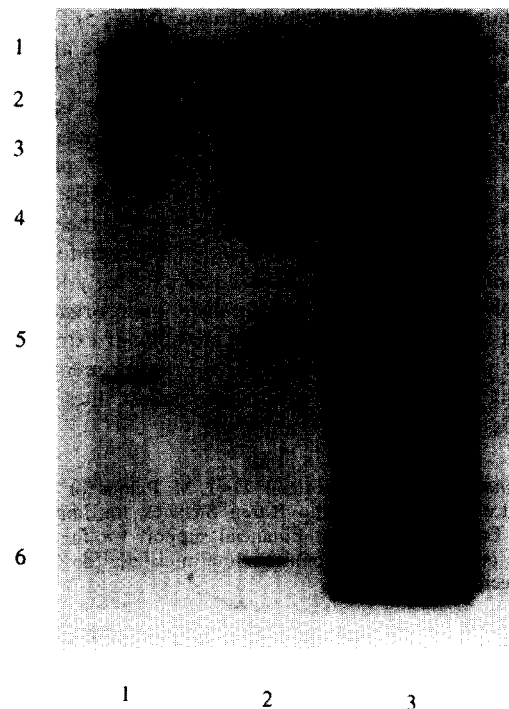


Fig. 5. Non-denaturing PAGE and SDS-PAGE of purified AM endoglucanase. Lane 1; SDS gel silver stained for proteins. Lane 2; SDS gel showing the silver-stained mol. wt calibration protein, 1 = phosphorylase b; 2 = BSA; 3 = ovoalbumin; 4 = carbonic anhydrase; 5 = soybean trypsin inhibitor; 6 = lysozyme. Lane 3; non-denaturing gel for endoglucanase activity stained with Congo red and destained as described in Material and Methods.

Table 1. Purification of endoglucanase from onion roots colonized by the arbuscular mycorrhizal fungus *Glomus mosseae*

Step	Total protein	Total activity	Specific activity	Yield	Purification
	(mg)	(units)	(units mg ⁻¹)	(%)	(x-fold)
Initial extract	44.4	559	12.6	100	1
Filtration	40.5	520	12.3	93	1
DEAE-Sephadex	16.7	400	27.5	71	2.1
Mono Q FPLC	4 × 10 ⁻²	4.2	105	0.7	8.3
Electroelution	6 × 10 ⁻⁴	1.5	2.500	0.2	198

For determination procedures see Materials and Methods.

to that of the external mycelium (Fig. 1, lane 5 and 3). Fractions with endoglucanase activity obtained by FPLC on the Mono Q column (peak M), were pooled, concentrated with a PM 10 membrane (Amicon) and applied to a PAGE, and then electroeluted from the polyacrylamide gel. The native mol. wt of the enzyme was determined by non-denaturing PAGE. An Mr of 29 kDa was obtained by comparison with markers of known mol. wt (Fig. 4). When the electroeluted fraction was analyzed by SDS-PAGE, a single polypeptide band was found. The relative mol. wt of the enzyme was determined with SDS-PAGE by comparison with markers of known mol. wt and an Mr value of 27 kDa was obtained for the endoglucanase (Fig. 5). These results suggest that the AM endoglucanase appears to be a monomeric protein.

The purification of AM endoglucanase is summarized in Table 1. Solutions of pure endoglucanase had a specific activity of 2500 units mg⁻¹ protein, and purification was 198-fold. The yield was 0.2%, and on the basis of fresh weight of roots, the purification yield was about 0.6 µg of pure endoglucanase. This amount of enzyme was extremely low, as might be expected from mutualistic microorganisms, and therefore the use of these methods of extraction and purification of endoglucanase do not seem sufficient for producing antibodies against these proteins.

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