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Polysaccharide hydrolases from Boscia senegalensis: purification and characterization of endo-1,3-beta-glucanase

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Polysaccharide Hydrolases from Leaves of *Boscia senegalensis*

Properties of Endo-(1→3)-β-D-Glucanase

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Abstract

The leaves of *Boscia senegalensis* are traditionally used in West Africa in cereal protection against pathogens, pharmacologic applications, and food processing. Activities of α-amylase, β-amylase, exo-(1→3, 1→4)-β-D-glucanase, and endo-(1→3)-β-D-glucanase were detected in these leaves. The endo-(1→3)-β-D-glucanase (EC 3.2.1.39) was purified 203-fold with 57% yield. The purified enzyme is a nonglycosylated monomeric protein with a molecular mass of 36 kDa and p*I* ≥ 10.3. Its optimal activity occurred at pH 4.5 and 50°C. Kinetic analysis gave V_{max}, k_{cat}, and K_m values of 659 U/mg, 395 s^{-1}, and 0.42 mg/mL, respectively, for laminarin as substrate. The use of matrix-assisted laser desorption ionization time-of-flight mass spectrometry and high-performance liquid chromatography revealed that the enzyme hydrolyzes not only soluble but also insoluble (1→3)-β-glucan chains in an endo fashion. This property is unusual for endo-acting (1→3)-β-D-glucanase from plants. The involvement of the enzyme in plant defense against pathogenic microorganisms such as fungi is discussed.

Index Entries: α-Amylase; β-amylase; β-glucanase; disodium 2,2'-bichinconitate; yeast glucan; high-performance liquid chromatography; matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

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Introduction

Boscia senegalensis is very well adapted to the semiarid and arid conditions that prevail over much of the African continent, with unusual drought and high-temperature resistance *(1,2)*. The physiologic behavior of the plant is extraordinary in its ability to stay evergreen in all hard Sahelian seasons. The importance of the plant for the rural agroeconomy in Africa has been illustrated by several reports, making it a plant of high interest *(1–4)*. The leaves of *B. senegalensis* are traditionally used for human and animal nutrition, protection of cereals against pathogens, and pharmacologic purposes *(2,4)*. It is well known that mixing a suspension of powder prepared from the leaves or roots with cereal flour (or porridge) results in sweet products *(2)*. The rationale behind the use of these leaves for the production of foods with improved taste (through probably the release of reducing sugars) has never been elucidated. The biocide activity of the leaves on insects has been justified by the presence of volatile compounds such as methylisothiocyanate and methylcyanide *(5)*. However, to our knowledge, no investigations on enzyme activities related to the properties of the plant have been conducted.

Quite a number of starch and β-glucan-degrading enzymes have been detected and purified from bacteria, fungi, and plants. The endo-acting β-D-glucanases from germinated grains and leaves of barley have been thoroughly investigated *(6)*. The most important classes are (1→3, 1→4)-β-D-glucanase (EC 3.2.1.73), (1→4)-β-D-glucanase (EC 3.2.1.4), and (1→3)-β-D-glucanase (EC 3.2.1.39). These cell-wall-degrading enzymes are involved in the catabolism of β-glucans during plant development. However, the level of (1→3)-β-D-glucanase in plants is higher than the activity required to depolymerize (1→3)-β-D-glucan deposits (minor polymer) *(7)*. Synthesis of (1→3)-β-D-glucanases is enhanced in response to pathogen infection or abiotic stress conditions *(8)*. However, only certain isoforms have a defensive role against injury *(9)*. Thus, (1→3)-β-D-glucanases have been suggested to be stress-related enzymes, playing a major role either in defense reactions against pathogens *(10)* or in a wide range of other attacks *(11–13)*. It has also been argued that the oligosaccharides released from plant or pathogen, by the action of (1→3)-β-D-glucanases, act as elicitors of the general immune system of the plant *(14)*. In recent years, chemically or enzymatically tailored fungal glucan found application in pharmacology as a stimulator and modulator of the immune system *(15,16)*. (1→3)-β-D-glucans are associated with antitumor, antibacterial, anticoagulatory, and wound-healing properties *(15)*; the soluble ones are the most active *(15)* and most suitable for pharmacologic applications *(16)*. It was therefore our challenge to search for a novel source of (1→3)-β-D-glucan-converting enzymes as a tool to modify these polymers. The present study aims to screen for glycosidase activities in the leaves of *B. senegalensis*, to elucidate how the plant improves the taste of processed cereal foods, and to isolate and characterize the most active β-D-glucanase.

Materials and Methods

Materials and Reagents

Laminarin (degree of polymerization of about 25) from *Laminaria digitata*, bovine serum albumin (BSA), phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), pullulan (from *Aureobasidium pullulans*), and Coomassie Brillant Blue G-250 were purchased from Sigma (St. Louis, MO). Pustulan (from *Umbilicaria popullosa*) was from Calbiochem (La Jolla, CA). CM-cellulose Akucell type AF 0305 was from Akzo Nobel (The Netherlands), and soluble starch, D-glucose, and D-mannose were from Merck (Darmstadt, Germany). Azurin-cross-linked-amylose (AZCL-amylose), *p*-nitro-phenyl-maltopentaoside (PNPG-5), and *p*-nitro-phenyl-α-D-glucoside were obtained from MegaZyme (Sydney, Australia). YM 10K membranes were purchased from Amicon (Danvers, MA) and barley (1→3, 1→4)-β-D-glucan from Biocon (Kilnagleary, Cork, Ireland). Baker's yeast (*Saccharomyces cerevisiae*) was obtained from Gist-Brocades (Delft, The Netherlands). Brewer's yeast (*S. cerevisiae*) was isolated as previously described by Konlani et al. *(17)*. Maltodextrins MD05 were obtained from Spreda (Burghof, Switzerland). Sorghum (1→3, 1→4)-β-D glucan was prepared as previously described by Verbruggen et al. *(18)*. Chemicals for electrophoresis, columns for chromatography, low molecular mass standard proteins (14.4–94 kDa), and p*I* standard proteins (p*I* 3.5–10.3) were supplied by Amersham Pharmacia Biotech (Uppsala, Sweden). Disodium 2,2'-bicinchoninate (BCA) was from Pierce. Water-insoluble yeast cell wall glucan (from *S. cerevisiae*) was generously provided by Professor F. M. Rombouts of the laboratory of Food Microbiology at Wageningen University (The Netherlands). All other chemicals were of analytical grade.

Plant Material

Green leaves of *B. senegalensis* (Pers.) Lam. ex. Poir (Capparaceae), harvested in the rainy season at Ouagadougou, were generously provided by the National Centre for Scientific and Technological Research of Burkina Faso. Healthy, undamaged leaves were selected and then thoroughly surface sterilized as described by Hrmova and Fincher *(12)* for barley leaves, but without the addition of nystatin. The leaves were dried by ventilation at room temperature (25–30°C) for 4–6 h, to a moisture content of approx 12%. The dried leaves were powdered with a micromill (Fritsch, Marius Instrumenten) and passed through a 0.5-mm sieve prior to enzyme extraction.

Enzyme Extraction

Unless indicated otherwise, all separation steps were carried out at 4°C. All buffers contained 0.02% (w/v) sodium azide to prevent microbial growth.

Powder (100 g) from the leaves of *B. senegalensis* was mixed with 200 mL of 0.050 *M* sodium acetate buffer (pH 5.0) containing 3 m*M* $CaCl_2$, 3 m*M* DTT, 0.1% (w/v) NaCl, and 3 m*M* PMSF for 10 min. The homogenate was centrifuged at 10,000*g* for 20 min, and the supernatant was used as

crude extract. The precipitate obtained between 20 and 70% of $(NH_4)_2SO_4$ saturation and was dissolved in extraction buffer. Insoluble materials were removed by centrifugation (10,000*g*, 10 min). After dialysis against the same buffer containing 3 m*M* $CaCl_2$ and 3 m*M* DTT, the material was lyophilized and kept at –20°C until further purification.

Purification of (1→3)-β-D-Glucanase

An aliquot of the lyophilized material was dissolved in 20 m*M* piperazine-HCl buffer (pH 6.0) containing 3 m*M* DTT and applied to an anion-exchange column (MonoQ HR 5/5, 5 × 50 mm, fast protein liquid chromatography [FPLC]) equilibrated with the same buffer. Proteins were subsequently eluted with a gradient of 20 m*M* piperazine-HCl (pH 6.0) containing 0.5 *M* NaCl and 3 m*M* DTT at 0.5 mL/min. The fractions with glucanase activity were applied to a cation-exchange column (MonoS HR 5/5, 5 × 50 mm, FPLC) equilibrated with 20 m*M* sodium acetate buffer (pH 5.0) containing 3 m*M* DTT. A linear gradient of 0–1 *M* NaCl in the same buffer was used to elute the proteins from the column at 1 mL/min. Active fractions were combined and concentrated by ultrafiltration using a YM 10K membrane (Amicon). The concentrate was applied on a gel filtration column (Superdex 75 pg, 60 × 16 mm, FPLC) equilibrated with 0.1 *M* sodium acetate buffer (pH 5.0) containing 0.150 *M* NaCl and 3 m*M* DTT at a flow rate of 0.8 mL/min. The elution of the proteins was monitored at 254 nm. Enzyme activities and protein contents were determined after each purification step. The native molecular mass of the enzyme was determined by Superdex 75-gel filtration chromatography, using the same conditions, after calibrating the column with low molecular mass protein standards.

Enzyme Assays and Determination of Protein

Enzymatic release of reducing sugars from glucan substrates was measured by the BCA reducing assay *(19,20)*, which was adapted for microtiter plates *(21)*. The standard assay for (1→3)-β-D-glucanase was performed by incubating the enzyme with laminarin (3 mg/mL) in 0.1 *M* sodium acetate (pH 4.5) in a microtiter plate at 40°C for 10 min. Reducing sugars were quantified by incubating 100 µL of the samples with 100 µL of the BCA reagent, and the mixture was heated at 80°C for 60 min. After cooling, the absorbance of the mixture was measured using an EAR-400 multiwell plate reader (SLT-Labinstruments, Australia). The control was prepared by adding the BCA reagent before the addition of the enzyme and subsequent heating of the mixture as described for the assay. One unit of enzyme activity was defined as the amount of enzyme releasing 1 µmol of glucose equivalent/min under assay conditions. The activities of β-amylase, α-amylase, and α-glucosidase were determined by using specific substrates as previously described *(22)*. Activity measurements were performed at least in triplicate. Total protein was quantified by the method of Sedmak and Grossberg *(23)* using BSA as standard protein.

Gel Electrophoresis

The purity and molecular mass of the enzyme were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a 10–15% polyacrylamide gradient gel under reducing conditions with the PhastSystem (Amersham Pharmacia Biotech). The gel was stained by Coomassie Brilliant Blue. Low molecular mass standard proteins (14.4–94 kDa) were used for calibration. The p*I* of the enzyme was estimated with the PhastSystem using standard proteins (p*I* 3.5–10.3). Proteins were detected by silver staining. Electrophoretic titration curves were performed in the same type of gels to monitor the purification procedure. All electrophoretic techniques were performed as described in the manufacturer's manual (Amersham Pharmacia Biotech).

Determination of Carbohydrate Content

The carbohydrate content of the purified enzyme preparation was determined by the resorcinol/H_2SO_4 acid method of Monsigny et al. *(24)* using D-mannose as well as D-glucose as standards. The standard sugars contained BSA at concentrations (50 µg/mL) comparable with the enzyme concentration in the test solutions in order to correct for the interference of the protein with the assay *(12)*.

Influence of pH and Temperature on Enzyme Activity and Stability

The effect of pH on the activity was measured by incubating laminarin (3 mg/mL) with the purified enzyme (0.1 U) in McIlvaine buffer (phosphate/citrate) over a pH range of 2.5–9.0 at 40°C for 10 min. The pH stability was determined by preincubating the enzyme in the same buffers at 20°C for 24 h. Afterward the pH was adjusted to 5.0 with 1 *M* acetate buffer (pH 5.0), and the residual activity was determined. For assaying residual enzyme activity, a pH of 5.0 was chosen because it appeared to be the optimal pH (after this experiment) and therefore was preferred instead of pH 4.5, used in the standard assay. The optimal temperature was determined by incubating laminarin (3 mg/mL) with the enzyme (0.1 U) in 0.1 *M* sodium acetate buffer (pH 5.0) at temperatures ranging from 20 to 80°C for 10 min. The temperature stability was studied by preincubating the enzyme in 0.1 *M* sodium acetate buffer (pH 5.0) at the same temperatures for 1 h. The residual enzyme activity was determined by incubating with laminarin (3 mg/mL) in 0.1 *M* acetate buffer (pH 5.0), at 40°C for 10 min. Activity measurements were performed in triplicate with the BCA reducing sugar assay, and controls were used for all assays.

Substrate Specificity and Action Pattern

The specificity of the enzyme for carbohydrate substrates was studied by measuring the release of reducing sugars after incubating the enzyme (0.5 U) with various polysaccharides (5 mg) in 0.1 *M* sodium acetate buffer (pH 4.5) (total volume of 1 mL) at 40°C for 30 min. After centrifuging the

incubation mixtures, the carbohydrates present in the supernatant were analyzed. Analytical high-performance anion-exchange chromatography (HPAEC) was performed on a Dionex Bio-LC high-performance liquid chromatography (HPLC) system (Sunnyvale, CA) as previously described *(22)*. Analytical high-performance size exclusion chromatography (HPSEC) was carried out on an SP8800 HPLC system (Spectra Physics, San José, CA) as previously described by Mutter et al. *(25)*. The matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis was performed with the Voyager-DE RP Biospectrometry Workstation (PerSeptive Biosystems, Framingham, Manchester, England) as described by Daas et al. *(26)*, using maltodextrins for calibration. Control assays in which the enzyme had been replaced by the buffer were used for all incubations.

Kinetic Properties

Kinetic parameters were determined by incubating the enzyme (0.5 U) and laminarin at concentrations ranging from 0.1 to 5 mg/mL in 0.1 *M* sodium acetate buffer (pH 4.5) at 40°C. The initial release of reducing sugars from laminarin was determined. For the calculation of the activation energy (E_a), the Q_{10} between 20 and 30°C was determined and the E_a calculated by the method of Segel *(27)* using the following equation:

$$E_a = 0.23RT_1T_2 \log(Q_{10})$$

in which R is the molar gas constant, and T_1 and T_2 are absolute temperatures. The assays were performed in triplicate using the BCA reducing sugar assay.

Measurement of Lytic Activity

A qualitative test to determine the lytic activity of the β-glucanase on viable yeast cells was performed by monitoring the reduction in optical density (OD) at 610 nm of exponentially growing *S. cerevisiae* cells as previously described by Nagata et al. *(28)*. The enzyme sample was desalted by dialysis and incubated with the cell culture in its exponential phase of growth at 40°C. The subsequent growth in the presence and absence of the enzyme was monitored by taking aliquots of the mixture at 1-h intervals for 24 h to measure the OD.

Results

Polysaccharide Hydrolases from Leaves of B. senegalensis

The crude extract of the leaves of *B. senegalensis* contained activities of α-amylase, β-amylase, exo-(1→3, 1→4)-β-D-glucanase, and endo-(1→3)-β-D-glucanase. The activities of these enzymes in the fraction obtained after protein precipitation by $(NH_4)_2SO_4$ (20–70% saturation), desalting, and lyophilization are given in Table 1. No α-glucosidase and endo-(1→3, 1→4)-β-D-glucanase activities were detectable with the assays used.

Table 1
Polysaccharide Hydrolases in 20–70% $(NH_4)_2SO_4$ Fraction Obtained from Leaves of *B. senegalensis*

Target enzyme	Substrate used	Specific activity (U/mg)[a]	Total activity (U/g of leaves)
α-Amylase	Azurin-crosslinked amylose	4.2 ± 0.2	12.8 ± 0.3
β-Amylase	*p*-nitrophenyl-maltopentaoside	51.7 ± 0.8	158.1 ± 4.2
α-Glucosidase	*p*-nitrophenyl-α-D-glucoside	0	0
Exo-(1,3; 1,4)-β-D-glucanase[b]	Barley (1,3; 1,4)-β-D-glucan	2.4 ± 0.1	7.3 ± 0.2
Endo-(1,3; 1,4)-β-D-glucanase	Barley (1,3; 1,4)-β-D-glucan	0	0
Exo-(1,3; 1,4)-β-D-glucanase	Sorghum (1,3; 1,4)-β-D-glucan	3.6 ± 0.1	11.0 ± 0.4
Endo-(1,3; 1,4)-β-D-glucanase	Sorghum (1,3; 1,4)-β-D-glucan	0	0
Endo-(1,3)-β-D-glucanase	Laminarin	8.9 ± 0.3	27.3 ± 1.0

[a]Total protein in the $(NH_4)_2SO_4$ fraction was approx 306 mg/100 g of leaves.

[b]The mode of action was analyzed by HPAEC; exoglucanases released only glucose monomers whereas endoglucanases released oligosaccharides with different degrees of polymerization. Total activity for polysaccharide hydrolysis was determined by the BCA assay.

Table 2
Purification of (1→3)-β-D-Glucanase from Leaves of *B. senegalensis*

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	1288	3476	2.7	1	100
$(NH_4)_2SO_4$ precipitation	306	2731	8.9	3.3	79
MonoQ	34	2597	75.5	9	75
MonoS	7.5	2294	306	113	67
Superdex 75	3.6	1977	549	203	57

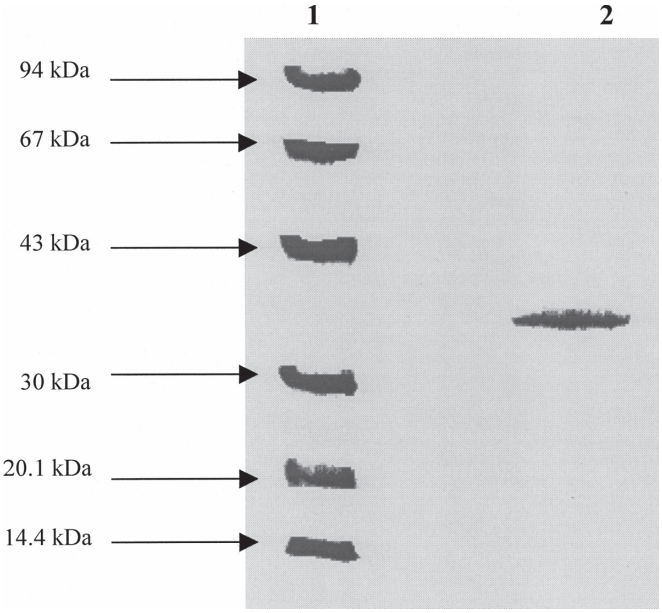

Fig. 1. SDS-PAGE of *B. senegalensis* (1→3)-β-D-glucanase on 10–15% PhastGel. Lane 1, low molecular weight markers; lane 2, purified enzyme.

Purification of (1→3)-β-D-Glucanase

Most of the (1→3)-β-D-glucanase was precipitated from the initial extract by 20–70% saturation with $(NH_4)_2SO_4$, increasing its specific activity 3.3-fold with 79% yield (Table 2). The fraction obtained between 20 and 70% $(NH_4)_2SO_4$ was desalted by dialysis and lyophilized without loss of activity. After application on a MonoQ column, approx 89% of the total protein was retained and 95% of the total (1→3)-β-D-glucanase activity was recovered. The fractions with β-glucanase activity were further separated on a MonoS column; two peaks with β-glucanase activity, representing 96 and 4% of the total activity, respectively, were obtained. The action patterns of the enzymes, as judged by HPAEC, revealed that both were (1→3)-β-D-glucan endohydrolases. The most abundant protein with endo-(1→3)-β-D-glucanase activity was further purified by gel filtration chromatography and characterized. The overall yield of the purification was approx 57% with a 203-fold purification, resulting in a final specific activity of 549 U/mg.

Physical Properties of (1→3)-β-D-Glucanase

After gel filtration chromatography, a single protein band with a molecular mass of 36 kDa was detected on SDS-PAGE under reducing conditions (Fig. 1). This is in good agreement with the native molecular mass (37 kDa) determined by gel filtration, confirming that *B. senegalensis* (1→3)-β-D-glucanase is a monomer. Isoelectric focusing and electrophoretic

Table 3
Substrate Specificity
of Purified *B. senegalensis* (1→3)-β-D-Glucanase Toward Various Glucans

Substrate	Major linkage types	Relative activity (%)
Laminarin	β-(1→3)	100
Insoluble yeast glucan (*S. cerevisiae*)	β-(1→3)	78
Pustulan	β-(1→6)	0
CM-cellulose	β-(1→4)	0
Barley glucan	β-(1→3, 1→4)	0
Sorghum glucan	β-(1→3, 1→4)	0
Starch	α-(1→4)	0
Pullulan	α-(1→6)	0

titration showed that the enzyme is a highly basic protein with p$I \geq 10.3$ (data not shown). No carbohydrate was detected in the enzyme preparation with the resorcinol/H_2SO_4 assay.

Effects of Temperature and pH on Enzyme Activity and Stability

The purified (1→3)-β-D-glucanase showed maximum activity for laminarin hydrolysis at about pH 4.5. The optimal temperature for activity of the enzyme toward laminarin was 50°C. When incubated for 24 h in McIlvaine buffers, at 20°C, the enzyme was stable in the range of pH 4.0–6.5. The optimum stability was about pH 5.0. The enzyme retained its full activity after incubation in 0.1 *M* sodium acetate buffer (pH 4.5) at 35°C for 1 h, in both the absence and presence of substrate. However, the enzyme gradually lost its activity at temperatures above 35–40°C. No residual activity could be detected after preincubation at 65°C for 1 h in the absence of substrate.

Kinetic Parameters

The enzymatic hydrolysis as a function of laminarin concentration followed Michaelis-Menten kinetics. A linear curve was obtained from Lineweaver-Burk plot for the hydrolysis of laminarin at concentrations of 0.1–5 mg/mL, yielding an apparent K_m value of 0.42 mg/mL, a V_{max} of 659 U/mg of protein, and a catalytic rate constant k_{cat} (turnover number) of 395 s^{-1}. The Q_{10} determined between 20 and 30°C was 1.3, and the calculated Arrhenius energy of activation (E_a) was 25 kJ/mol for laminarin hydrolysis.

Substrate Specificity

The enzyme specifically hydrolyzed molecules containing contiguous (1→3)-β-D-glucosidic bonds, such as laminarin and yeast glucan (Table 3). Indeed, *L. digitata* laminarin and yeast glucan are essentially linear (1→3)-β-D-glucans with a low degree of glucosyl substitution at O-6 *(16)*. The level of (1→3)-β-linked glucosyl units is up to 80% *(16)*. As shown in Table 3, no

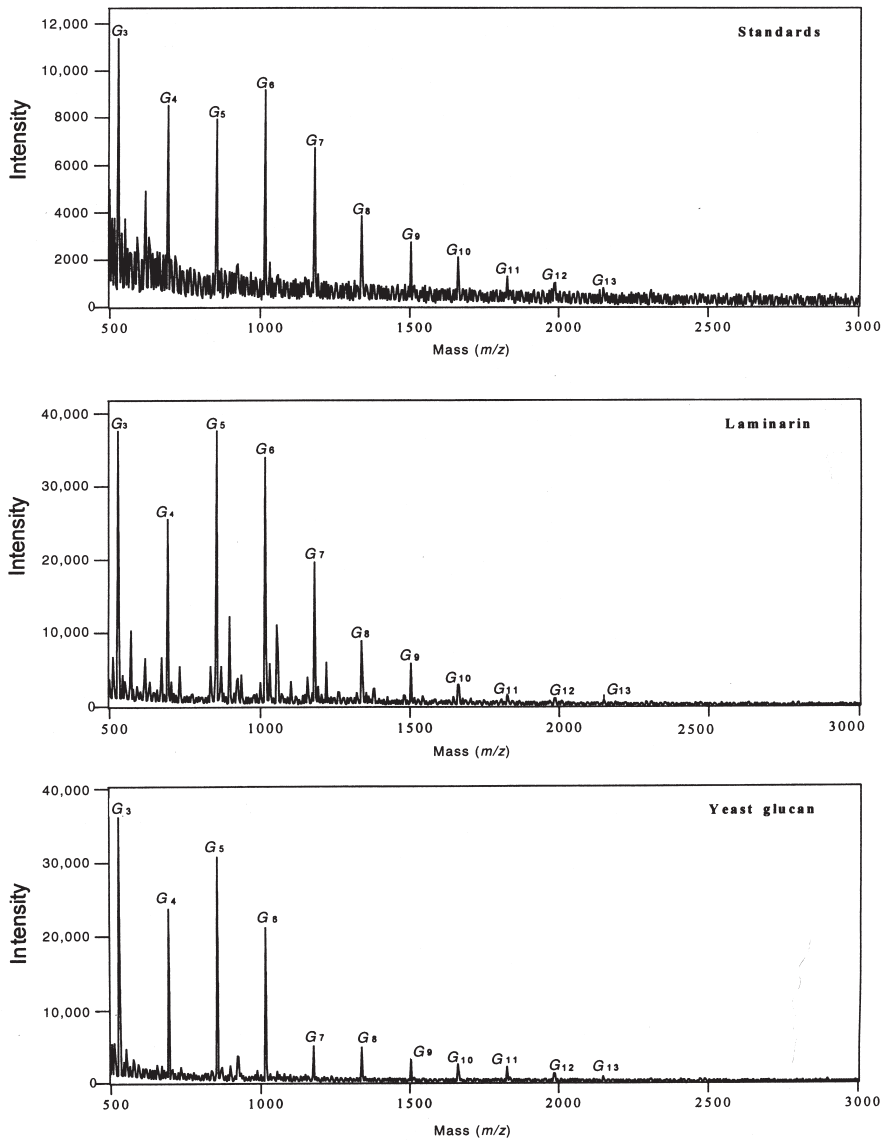

Fig. 2. Product analysis by MALDI-TOF-MS after the action of (1→3)-β-D-glucanase on laminarin or yeast glucan. Standard glucose oligomers of known degree of polymerization were used for calibration. G_n, glucose oligomers (n = degree of polymerization).

activity was detected toward CM-cellulose, pustulan, (1→3, 1→4)-β-D-glucan from barley and sorghum, starch, and pullulan.

Action Pattern

The degree of polymerization of the released products after enzyme incubation was determined by MALDI-TOF-MS using glucose oligomers as standards (Fig. 2). The hydrolysis of laminarin and yeast glucan pro-

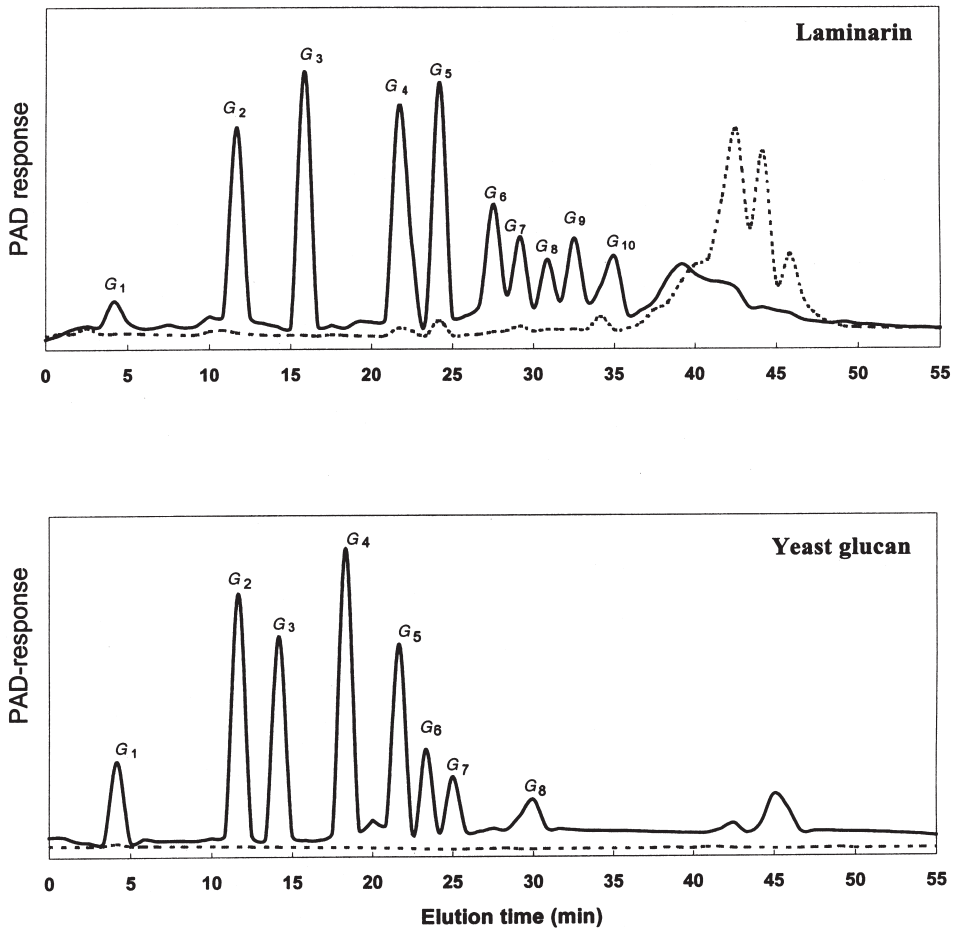

Fig. 3. Product analysis by HPAEC after the action of (1→3)-β-D-glucanase on laminarin or yeast glucan. G_n, glucose oligomers (n = degree of polymerization). (– – –), Control without enzyme; (——), substrate + enzyme.

duces oligosaccharides with degree of polymerization up to 13 (Fig. 2). These results indicate the endohydrolytic action of the purified (1→3)-β-D-glucanase. The HPSEC analysis of the products released from laminarin in time showed a gradual shift of the molecular mass of the initial substrate with a simultaneous production of oligomers (not shown). It also showed that no more laminarin was present after 30 min of incubation with the enzyme. In the supernatant of the yeast glucan, no oligosaccharides were detectable before enzyme incubation, as analyzed by HPAEC, HPSEC, and MALDI-TOF-MS. However, after enzymatic hydrolysis, soluble oligomers were released. The retention time of laminarin oligosaccharides on HPAEC, being a function of their degree of polymerization *(29)*, allowed the low–degree of polymerization oligosaccharides to be determined (Fig. 3). Although the factors' response of the various oligosaccharides is different, the major hydrolysis products, as judged by the combination of MALDI-

TOF-MS (Fig. 2) and HPAEC (Fig. 3) patterns, were apparently G_2–G_6 for both laminarin and yeast glucan.

Growth Inhibition of Viable Yeast Cells

In a qualitative test, it was shown that the growth of two species of *S. cerevisiae* (baker's and brewer's yeast) was inhibited by the addition of the purified endo-(1→3)-β-D-glucanase. In the presence of enzyme, the absorbance at 610 nm had decreased to about 33% of the absorbance of the control experiment without the enzyme. This was essentially the case for the whole growth period (24 h, pH 6.0, 40°C).

Discussion

We have shown the presence of several carbohydrate hydrolases in the leaves of *B. senegalensis*. These enzymes justify the traditional use of this plant material in food processing *(2)*. Enzymes in extract of the leaves could be the basis for application in the production of cereal-based flour, in order to increase the energy intake of infants by reducing the dietary bulk of cereal porridges (unpublished results). They could be an alternative for the currently used amylases from microorganisms and barley malt *(30)*. The production of sweet products after incubation of the leaves with cereal flour can be explained by the conversion of starch and cell-wall β-glucans into fermentable sugars by the glucosyl hydrolases present in the leaves. The depolymerization of β-glucans and starch during the preparation of sorghum beer may improve the filtration of wort *(31)* and the production of fermentable sugars; this may also explain the traditional use of *B. senegalensis* leaves in sorghum beer manufacturing. The specific activity (2.7 U/mg) of the (1→3)-β-D-glucanase in the crude extract of the leaves of *B. senegalensis* is about three times higher than that of the homologous enzyme in the leaves of barley *(12)*. The relatively high activity of the endo-(1→3)-β-D-glucanase and its possible involvement in the resistance of the leaves against pathogens have motivated its purification and biochemical characterization.

The basic 36-kDa (1→3)-β-D-glucan endohydrolase purified from the leaves of *B. senegalensis* has a molecular mass similar to (1→3)-β-D-glucanase from cucumber leaves *(32)*, but a higher molecular mass than endo-(1→3)-β-D-glucanases from the leaves of barley *(12)*, rice *(33)*, and tomato *(34)*. Several highly basic (1→3)-β-D-glucan endohydrolases (p*I* 8–11) from either plants *(7,12,33)* or microbial origin *(35)* have been reported in the literature. The resorcinol/H_2SO_4 assay indicated that the purified enzyme is not glycosylated or that the glycosylation level is too low to be detected under the assay conditions used. Both glycosylated and nonglycosylated (1→3)-β-D-glucanases have been found in barley leaves *(12)*.

The enzyme resembles other plant enzymes with respect to pH and temperature optima *(12,34,36)*, but it is relatively more thermostable than the (1→3)-β-D-glucanases from the leaves of barley *(12)*, tomato *(34)*, and

rice *(33)*. The K_m for laminarin (0.42 mg/mL) is substantially lower than reported for (1→3)-β-D-glucanases from other plants *(12,34,36)*. The relatively low value of K_m indicates that the enzyme has a high affinity for its substrate. The turnover number (k_{cat} = 395 s^{-1}) is higher than the values reported for the (1→3)-β-D-glucanase isoenzymes from barley leaves *(12)*. The activation energy (E_a = 25 kJ/mol) is in the range of most carbohydrate hydrolases. It is important to realize that kinetic parameters of polysaccharide hydrolases are approximate values because of the heterogeneity in size of the substrates and because hydrolysis products can become additional substrates *(12)*.

The carbohydrates used to determine the specificity of the enzyme for its substrate were either linear or branched. The substrate specificity and action pattern of *B. senegalensis* (1→3)-β-D-glucanase showed that the enzyme splits, in an endo fashion, exclusively contiguous (1→3)-β-glucosyl linkages in polysaccharides. It is well known that the elution of linear oligosaccharides (of the same monomer) from Dionex CarboPac columns is correlated with their degree of polymerization *(26,37,38)*. Moreover, using HPAEC low–degree of polymerization laminarin oligosaccharides elute as a function of their degree of polymerization *(29)*. However, for branched oligosaccharides with a high degree of polymerization, the assignment of the peaks is much more difficult because the degree of ionization (and therefore the binding) is more complex. The difference in elution patterns on HPAEC analysis shows that differently branched products are present after hydrolysis of laminarin and yeast glucan. The ratio of the (1→3)-β to (1→6)-β linkages in laminarin (from *L. digitata*) and yeast glucan is 7:1 *(39)* and 4:1 *(40)*, respectively. In other words, the degree of substitution of the (1→3)-β main chain by the (1→6)-β-type side chains is higher in yeast glucan than in laminarin. In addition, the degree of polymerization of the side chains is higher in yeast glucan. Therefore, the oligosaccharides released from yeast glucan have a more branched character than those from laminarin. These data could explain the observed differences in retention times of glucose oligomers of the same degree of polymerization on HPAEC. The HPSEC analysis was useful to show the complete degradation of laminarin and also the solubilization of yeast glucan after the enzyme's action. Nevertheless, the high sensitivity of the MALDI-TOF-MS allowed more accurate identification of the degree of polymerization of the products released after the action of the enzyme on the polysaccharides. Based on the substrate specificity and action pattern, the enzyme isolated from the leaves of *B. senegalensis* could therefore be classified as a (1→3)-β-D-glucan glucanohydrolase (EC 3.2.1.39).

In contrast to the (1→3)-β-D-glucanases from plants *(12,13)*, which hardly hydrolyze insoluble *S. cerevisiae* β-glucan, the (1→3)-β-D-glucanase from *B. senegalensis* split both soluble and insoluble substrates to almost the same extent. The purified enzyme not only showed "solubilization activity" in an endo manner on isolated insoluble yeast glucan, but it also inhibited the growth of living yeast cells in the absence of other lytic components

such as proteases or chitinases. These results indicate that the enzyme has a potential lytic activity toward microorganisms with cell walls composed of (1→3)-β-D-glucans. Lytic endo-(1→3)-β-D-glucanases have been found in microorganisms *(28,35)* and pathogen-infected plants *(10)*, but not in healthy naturally occurring plants. The property of the *B. senegalensis* enzyme differs from other plant endo-(1→3)-β-D-glucanases characterized so far in its ability to hydrolyze yeast cell-wall glucan. The relatively abundant appearance of the enzyme in the leaves of *B. senegalensis* and its biochemical properties could make it a candidate for applications such as the biotechnologic conversion of (1→3)-β-D-glucans for food and pharmacologic applications.

The property of the leaves of *B. senegalensis* to protect cereals against pathogens is well known *(2,4,5)*. Their biocide activity on insects has been justified by the presence of methylisothiocyanate and methylcyanide *(5)*. In addition to those compounds and because of the structural similarities to fungal and yeast glucan *(15,16)*, the purified glucanase in the present work could also play a role in the resistance of the plant against fungal invasions. This enzyme could function as part of a defense strategy, which is not elicited by pathogen invasion *(7,12)*. The enzyme could be associated with the functions of plant cell-wall degradation as well as a preemptive role of protection. Indeed, it is known that (1→3)-β-D-glucan endohydrolases participate in the defense process against pathogenic fungi by causing extensive breakdown of their cell walls, which are constituted in majority by (1→3)- and (1→3, 1→6)-β-glucans *(41)*.

The fact that *B. senegalensis* preserves its leaves in a green state during all the drastic seasons in the semiarid and arid environments of Africa *(1,2)* may be related to various defense mechanisms developed by the plant to resist injuries, such as the synthesis of a lytic (1→3)-β-D-glucan endohydrolase. This hypothesis is further strengthened by recent works giving a body of evidence that the (1→3)-β-D-glucan endohydrolase is expressed more in the leaves of tomato *(42)* and wheat *(43)* resistant to fungi than in the susceptible ones.

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