Purification and Some Properties of a Novel Endo- β -(1 $\rightarrow$ 6)-D-galactanase from *Aspergillus niger*

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An endo- β -(1 $\rightarrow$ 6)-D-galactanase has been purified 26-fold (14% activity yield) from a crude enzyme preparation from *Aspergillus niger*. The enzyme was homogeneous on PAGE and SDS-PAGE, with an apparent molecular mass of 60 kDa. It has maximum activity at pH 3.5 on an enzymatically desarabinosylated grape 3,6-galactan-protein (Gp), being unreactive towards the native grape AGp. The enzyme liberated β -(1 $\rightarrow$ 6)-D-galactobiose and galactose in a final 1:0.26 molar ratio, β -(1 $\rightarrow$ 3)-D-galactosidic bonds being resistant to the action of enzyme.

Type II arabino-3,6-galactan-proteins (AGPs) are found in numerous higher plants¹⁾ and although the structure of their carbohydrate moiety is rather well known,^{2–4)} the overall organization of these macromolecules is not fully understood, especially the distribution and degree of polymerization (DP) of (1 $\rightarrow$ 3) and (1 $\rightarrow$ 6)-linked galactose chains. AGPs are hydrolyzable by several enzymes including α -L-arabinofuranosidases (EC 3.2.1.55),^{4–6)} and exo- and endo- β -D-galactanases.^{4,6,7)} An endo-galactanase has been partially purified from a koji culture of *Rhizopus niveus*⁷⁾ and liberated from coffee bean arabinogalactan⁸⁾ arabinose, galactose, and β -(1 $\rightarrow$ 6)-D-galactobiose, but the enzyme was possibly contaminated by an α -L-arabinofuranosidase due to liberation of arabinose and was not unambiguously demonstrated to be homogeneous. Another galactanase from *Irpex lacteus*⁹⁾ was similarly reported as acting on a radish seed AGp,³⁾ but neither complete purification procedure nor extensive characterization have been reported. An exo-galactanase has been purified from radish seeds,⁶⁾ cleaving both β -(1 $\rightarrow$ 3) and β -(1 $\rightarrow$ 6)-D-galactooligomers into galactose and pref-

erentially splitting β -(1 $\rightarrow$ 6)-D-galactopyranosidic bonds in desarabinosylated radish AGPs. More recently, Tsumuraya *et al.*⁴⁾ obtained an exo- β -(1 $\rightarrow$ 3)-D-galactanase from *Irpex lacteus*, which specifically cleaves β -(1 $\rightarrow$ 3)-D-galactopyranosidic linkages in β -(1 $\rightarrow$ 3)-D-galactooligosaccharides with liberation of galactose. It degrades a desarabinosylated radish root AGp into galactose, β -(1 $\rightarrow$ 6)-D-galactooligosaccharides of DP 2–5, and their corresponding acidic oligomers carrying 4-O-methyl-glucuronic acid as non-reducing terminals. The enzyme has a unique mode of action since it degrades β -(1 $\rightarrow$ 3)-D-galactan chains into galactose by by-passing branching points, thus liberating intact side-chains carrying an additional D-galactosyl residue at the reducing end.

We now report on the purification and characterization of a novel endo- β -(1 $\rightarrow$ 6)-D-galactanase from *Aspergillus niger* which degrades only β -(1 $\rightarrow$ 6)-D-galactosidic bonds in desarabinosylated AGp from grape berries.

Materials and Methods

Source of enzyme. A crude liquid enzyme preparation from *Aspergillus niger* (Arabana 8001/8002) was obtained

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from Gist-Brocades France (Seclin, France).

Chemicals and enzymes. Phenyl Sepharose CL-4B, DEAE-Sephacel, Sephacryl S-200 HR, Epoxy-activated Sepharose 6B, PBE 94, Polybuffer 74, and marker proteins for SDS-PAGE were from Pharmacia LKB Biotechnology AB (Uppsala, Sweden). Bio-Gel P-2 was from Bio-Rad (Richmond, CA., U.S.A.). Crude arabinan was obtained from pea cotyledon cell walls by extraction ($4\times$) with 2% EDTA sodium salt (pH 5.0) at 100°C for 4 hr^{10} and used as it is. The Sepharose 6B-arabinan was prepared strictly as described by Waibel *et al.*¹¹ β -(1 $\rightarrow$ 6)-D-galactobiose was purchased from Sigma Chemical Co. (St. Louis, U.S.A.). *p*-Nitrophenyl glycosides and polysaccharides used for detection of contaminating hydrolases in the purified enzyme have been previously described.¹² All other chemicals were of the highest available quality.

α -L-arabinofuranosidase (EC 3.2.1.55) was purified from a culture supernatant of the white-rot fungus *Dichomitus squalens* (426 nkat/mg; 1 nkat = 1 nmol of arabinose liberated per second from a 2 mM *p*-nitrophenylarabinofuranoside solution, pH 4.2, at 40°C).¹² β -nicotinamide-adenine dinucleotide (NAD^{+}), β -D-galactosidase (EC 3.2.1.23) from *Escherichia coli*, and β -D-galactose dehydrogenase (EC 1.1.1.48) from *Pseudomonas fluorescens*, and marker proteins for Sephacryl S-200 HR calibration were from Boehringer Mannheim (Germany).

Substrate of the enzyme. The substrate was prepared by enzymatic desarabinosylation of a neutral arabino-3,6-galactan-protein (AGp) from the pulp of grape berries⁵ as follows: 100 mg of AGp were dissolved in 20 ml of 0.1 M acetate buffer (pH 4.2), and 400 μl of α -L-arabinofuranosidase (250 nkats) were added. After 24 hr hydrolysis at 40°C , the digestion medium was placed in a boiling water bath for 5 min, extensively dialyzed against distilled water, concentrated under reduced pressure, and freeze-dried. The 3,6-galactan-protein (Gp) (yield 70%) had a galactose content of 80%, of which $\sim 50\%$ as β -(1 $\rightarrow$ 6)-D-galactosyl moieties (including terminal non-reducing galactose).⁵

Enzyme assay. The β -(1 $\rightarrow$ 6)-D-galactanase activity was assayed by measuring the amount of β -(1 $\rightarrow$ 6)-D-galactooligosaccharides (mainly galactobiose) liberated from the grape galactan after complete hydrolysis of oligomers by β -D-galactosidase from *Escherichia coli* and subsequent measurement of galactose by β -D-galactose dehydrogenase from *Pseudomonas fluorescens*.¹³ The reaction was done by incubating 25 μl of a 2% galactan solution in 0.1 M acetate buffer (pH 4.2), and 5 μl of enzyme ($<10^{-2}$ nkat) at 40°C for 20 min. The reaction medium was passed for 5 min in a boiling water bath, then 7 μl of 1 M phosphate buffer (pH 7.2), and 3 μl of β -D-galactosidase were added. After 30 min at 40°C , 840 μl of 0.1 M Tris-HCl buffer (pH 8.6), 100 μl of 5 mM NAD^{+} solution in water

and 20 μl of β -D-galactose dehydrogenase were added. After 90 min at room temperature, the absorbance at 340 nm was measured. One nkat of enzyme activity is defined as the activity liberating 1 nmol of equivalent β -(1 $\rightarrow$ 6)-D-galactobiose per second from the grape galactan at 40°C (pH 4.2).

Contaminating activities were tested as formerly described (17 hr, 40°C).¹² using the same concentration of enzyme as in the standard activity assay.

Mode of action. Fifteen mg of the grape 3,6-galactan-protein were dissolved in 7 ml of 0.1 M acetate buffer (pH 4.2), 350 μl of the enzyme (9 nkats) were added, and the medium was incubated at 40°C . Samples (1 ml) were withdrawn at 0, 10 min, 30 min, 3 hr, 18 hr, and 46 hr, and injected onto a column (1.2 $\times$ 150 cm) of Bio-Gel P-2 at 6 ml/hr using water as eluant. Oligosaccharides were detected in effluents with an Erma ERC-7512 differential refractometer. Hydrolysates were also analyzed by thin layer chromatography.

Analysis of reaction products. Oligosaccharides liberated by the enzyme were separated by thin layer chromatography on Kieselgel 60 plates (Merck, Darmstadt, Germany), using acetonitrile-water (85:15)($2\times$) and detected by spraying 0.2% naphthoresorcinol in ethanol-sulfuric acid (95:5) and heating (100°C , 10 min). Identification of the major reaction product [β -(1 $\rightarrow$ 6)-D-galactobiose] was done after purification by gel permeation chromatography on Bio-Gel P-2, by methylation with methyl iodide,¹⁴ acid hydrolysis, alditol acetate derivatization, and GC-MS.^{15,16} The disaccharide was also converted into its trifluoroacetate derivative and analyzed by GC-MS.¹⁷ Finally, it was chromatographed on an HPTLC Kieselgel 60 plate using acetonitrile-water (85:15)($3\times$; 1st dimension) and boric acid-*n*-butanol-isopropanol (2:3:5)($2\times$; 2nd dimension). All these identification techniques were also applied in parallel on the β -(1 $\rightarrow$ 6)-D-galactobiose standard.

Measurement of protein. Protein was measured by the method of Lowry *et al.*¹⁸ with preliminary precipitation with 10% trichloroacetic acid using ovalbumin as a standard.

Measurement of molecular weight.

(i) **Gel filtration on Sephacryl S-200 HR.** The galactanase was passed at 40 ml/hr through a column (1.6 $\times$ 95 cm) of Sephacryl S-200 HR equilibrated with 50 mM phosphate buffer (pH 7.2) containing 0.3 M NaCl. The galactanase elution volume ($A_{280\text{ nm}}$) was confirmed by an activity assay.

(ii) **Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.** SDS-PAGE using 5% stacking and 12.5% resolving gel of 1.5 mm thickness was done by the method of Laemmli¹⁹ and proteins were stained with either Coomassie brilliant blue R-250 or silver nitrate.²⁰ The

galactanase was also electrophoresed by PAGE using a 7% resolving gel without SDS at pH 8.3.

Results

Purification of enzyme

The whole purification procedure was done at 4°C. The purification steps are summarized in Table I. Solid ammonium sulfate (5.05 g) was gradually added to 10 ml of the crude enzymatic preparation, with mechanical stirring, to 90% saturation, and the solution was left overnight at 4°C. The precipitate was collected by centrifugation and repeatedly washed with 90% ammonium sulfate with intermittent centrifugation. The pellet was dissolved in 4 ml of 50 mM phosphate buffer (pH 7.2), containing 1 M ammonium sulfate, and the solution was put on a column (2.6 $\times$ 24 cm) of Phenyl Sepharose CL-4B

equilibrated with the same buffer (flow rate 50 ml/hr). The unbound proteins were recovered, dialyzed against 50 mM imidazole-HCl buffer (pH 7.2), and put on a column (2.6 $\times$ 9 cm) of DEAE-Sephacel equilibrated in the same buffer. The column was washed with two column volumes, and the enzyme was eluted with a linear NaCl concentration gradient, at a flow rate of 50 ml/hr. The linear gradient was made up of four column volumes, two of 50 mM imidazole-HCl buffer (pH 7.2), and two of 0.4 M NaCl in the same buffer. The active fractions (Fig. 1) were pooled, dialyzed against 50 mM phosphate buffer (pH 7.2) containing 0.3 M NaCl, concentrated through a dialysis bag with Sephadex G 200 to a volume of 1 ml, and put on a column (1 $\times$ 70 cm) of Sephacryl S-200 HR equilibrated in the same buffer at a flow rate of 16 ml/hr. The active fractions (Fig. 2) were pooled, dialyzed against

Fig. 1. Ion-exchange Chromatography of Endo- β -(1 $\rightarrow$ 6)-D-galactanase on DEAE-Sephacel.

The experimental conditions are given in the text. (—), protein $A_{280\text{nm}}$; (—●—), endo- β -(1 $\rightarrow$ 6)-D-galactanase activity; (----), NaCl concentration.

Fig. 2. Size Exclusion Chromatography of Endo- β -(1 $\rightarrow$ 6)-D-galactanase on Sephacryl S-200 HR.

The experimental conditions are given in the text. (—), protein $A_{280\text{nm}}$; (—●—), endo- β -(1 $\rightarrow$ 6)-D-galactanase activity.

Table I. PURIFICATION OF ENDO- β -(1 $\rightarrow$ 6)-D-GALACTANASE FROM *Aspergillus niger*

	Total protein (mg)	Total activity (nkat)	Specific activity (nkat/mg)	Yield (%)	Purification
Crude enzyme	310	—	—	—	—
Ammonium sulfate	179	—	—	—	—
Phenyl Sepharose CL-4B	119	—	—	—	—
DEAE-Sephacel	23	364	16	100	1
Sephacryl S-200 HR	4.8	227	48	62	3
DEAE-Sephacel	3.4	216	64	59	4
Arabinan-Sepharose 6B	1.5	157	105	43	6.6
PBE 94	0.12	50	420	13.7	26.2

50 mM imidazole-HCl buffer (pH 7.2), and put on a column (2.6 × 2 cm) of DEAE-Sephacel equilibrated with the same buffer, and bound enzyme was released by a one-step application of 0.26 M NaCl in 50 mM imidazole-HCl buffer (pH 7.2). After extensive dialysis against phosphate buffer (pH 7.2) containing 0.5 M KCl, the enzyme was put on a column (1 × 8 cm) of Sepharose 6B-arabinan equilibrated with the same buffer. Unbound proteins were recovered, dialyzed against 25 mM histidine-HCl buffer (pH 6.2), and injected at 28 ml/hr onto a column (1 × 30 cm) of PBE

94 equilibrated with the same buffer. After extensive washing with two column volumes, 220 ml of Polybuffer 74 (dilution 8) adjusted to pH 4.0 with 4 M HCl were passed through the column at the same flow rate, followed by 20 ml of 25 mM acetate buffer (pH 4.2) containing 0.1 M NaCl. Finally the enzyme was eluted by a linear gradient (0.1 → 0.4 M; 3 column volumes) of NaCl in 25 mM acetate buffer (pH 4.2). Active fractions were pooled (Fig. 3) and the purified enzyme was then extensively dialyzed against 50 mM acetate buffer containing 0.02% sodium azide.

At the final stage, the enzyme was purified about 26-fold and had a specific activity of 420 nkat/mg (activity recovery ~14%).

Fig. 3. Chromatofocusing of Endo- β -(1→6)-D-galactanase on PBE 94.

The experimental conditions are given in the text. (—), protein $A_{280\text{nm}}$; (—●—), endo- β -(1→6)-D-galactanase activity.

Homogeneity and molecular weight

The enzyme had no detectable activity against *p*-nitrophenyl glycosides and polysaccharides¹²⁾ after 17 hr at 40°C. The purified enzyme yielded a single band of 60 kDa apparent molecular mass in SDS-PAGE when stained by Coomassie brilliant blue R-250, few additional very faint bands appearing after silver staining (Fig. 4). PAGE gave the same result (not shown). The relative molecular mass was 29 kDa by gel filtration on a calibrated Sephacryl S-200HR column (Fig. 5).

Fig. 4. Estimation of Molecular Mass of Endo- β -(1→6)-D-galactanase by SDS-PAGE.

The purified enzyme was heated for 5 min at 100°C in 0.1 M Tris-HCl buffer (pH 6.9) containing 1% sodium dodecyl sulfate, 5% 2-mercaptoethanol, and 5% glycerol, and electrophoresed by the method of Laemmli.¹⁹⁾ Proteins were stained by Coomassie brilliant blue R-250 (10 μ g—lane 1) or by silver staining²⁰⁾ (0.2 μ g—lane 2). Marker proteins were: (1) phosphorylase B (94 kDa), (2) bovine serum albumin (67 kDa), (3) ovalbumin (43 kDa), (4) carbonic anhydrase (30 kDa), (5) trypsin (20.1 kDa), and (6) α -lactalbumin (14.4 kDa). (●), endo- β -(1→6)-D-galactanase.

Fig. 5. Estimation of Molecular Mass of Endo- β -(1 $\rightarrow$ 6)-D-galactanase by Gel Filtration on Sephacryl S-200 HR.

The experimental conditions are given in the text. Marker proteins were: (1) bovine serum albumin (68 kDa), (2) ovalbumin (45 kDa), (3) chymotrypsinogen A (25 kDa), and (4) cytochrome (12.5 kDa). (●), endo- β -(1 $\rightarrow$ 6)-D-galactanase.

Fig. 6. Effects of pH on the Activity and Stability of Endo- β -(1 $\rightarrow$ 6)-D-galactanase.

(—□—): The enzyme activity was measured under standard conditions in universal buffer from pH 2.5 to 9.0 (—■—): The enzyme stability was tested under standard conditions after incubation for 2 hr at 40°C in universal buffer from pH 3.5 to 8.0.

Properties of the enzyme

The optimum pH of the enzyme was 3.5 (Fig. 6) at 40°C in a 20-min assay, 85% of maximum activity being observed at pH 2.5. The enzyme was quite stable in acidic media for 2 hr at 40°C (Fig. 6), the activity decreasing strongly above pH 6.2.

The optimum reaction temperature was 60°C at pH 4.2 in a 20-min assay (Fig. 7) and the enzyme was thermostable up to 30°C (Fig. 7)

Fig. 7. Effects of Temperature on the Activity and Stability of Endo- β -(1 $\rightarrow$ 6)-D-galactanase.

(—□—): The enzyme activity was measured under standard conditions at various temperatures from 23°C to 80°C. (—■—): The enzyme stability was tested under standard conditions after incubation in 0.1 M acetate buffer (pH 4.2) at various temperatures from 10°C to 60°C.

Fig. 8. Thin Layer Chromatogram of the Enzymatic Hydrolysates of 3,6-Galactan-protein from Grape Berries by Endo- β -(1 $\rightarrow$ 6)-D-galactanase.

The experimental conditions are given in the text. Lane 1, galactose; lane 2, β -(1 $\rightarrow$ 6)-D-galactobiose; lanes 3 $\rightarrow$ 8, hydrolysates at 0, 10 min, 30 min, 3 hr, 18 hr, and 46 hr.

retaining 17% of activity after 4 hr at 60°C at pH 4.2.

The galactanase had a typical Michaelis-Menten saturation curve, the K_m for the des-arabinosylated grape 3,6-galactan-protein be-

Fig. 9. Chromatography on Bio-Gel P-2 of Oligosaccharides Released from 3,6-Galactan-protein from Grape Berries by Endo- β -(1 $\rightarrow$ 6)-D-galactanase.

The experimental conditions are given in the text. (A), hydrolysate at 3 hr; (B), at 46 hr.

Fig. 10. Course of Liberation of β -(1 $\rightarrow$ 6)-D-Galactobiose and Galactose by the Action of Endo- β -(1 $\rightarrow$ 6)-D-galactanase on 3,6-Galactan-protein from Grape Berries. (—○—), β -(1 $\rightarrow$ 6)-D-galactobiose (Gal_2); (—●—), galactose (Gal).

ing 4.5 mg/ml and the maximum velocity, $V_{\max}$, 500 nkat/mg of enzyme.

Mode of action

The oligosaccharides liberated by the galactanase from the 3,6-galactan-protein were separated by thin layer chromatography (Fig.

8) and size exclusion chromatography on Bio-Gel P-2 (Fig. 9). β -(1 $\rightarrow$ 6)-D-galactobiose was the major hydrolysis product from the very beginning of the reaction, galactose being produced in a lower proportion. Higher oligomers up to DP 7 were also liberated in small amounts (Fig. 9a) and rapidly disappeared from the reaction medium (Fig. 9b). As the reaction proceeded, galactobiose (Gal_2) and galactose (Gal) were liberated (Fig. 10), corresponding in the end (addition of extra enzyme without effect) to 43% of the galactose contained in the polymer (Gal_2/Gal molar ratio = 1 : 0.26).

Discussion

No natural substrate- β -(1 $\rightarrow$ 6)-D-galactan was available for measuring the activity of the enzyme, so we used an enzymatically des-arabinosylated arabino-3,6-galactan-protein from grape pulp. However, due to scarcity of this substrate, we established a micro-enzymatic method for activity measurement, instead of the substrate-consuming Nelson procedure.²¹⁾ We have checked that both β -D-galactosidase from *E. coli* and β -D-galactose dehydrogenase from *P. fluorescens* had not activity by themselves on the substrate. Since a β -D-galactosidase was present in the crude enzyme preparation, it was not possible to measure the endo- β -(1 $\rightarrow$ 6)-D-galactanase activity in the first steps of purification.

β -D-galactosidase was almost fully adsorbed onto Phenyl Sepharose CL-4B and remaining traces were eliminated after ion-exchange chromatography on DEAE-Sephacel. Similarly, the α -L-arabinofuranosidase present in the crude enzyme was totally fixed onto the Sepharose 6B-arabinan. The enzyme must be acidic in nature ($\text{pI} < 4.0$) since it was not eluted in the pH gradient when chromatofocusing on PBE 94, and was ultimately purified by applying a NaCl concentration gradient on this gel. The observed discrepancy between SDS-PAGE and size exclusion chromatography molecular mass determinations could be due to the glycosylated nature of the enzyme as

demonstrated by its positive interaction with Concanavalin A-Sepharose. The purified enzyme was inactive on the native AGp (17 hr, 40°C) without preliminary enzymatic desarabinosylation similarly to the exo-galactanase from radish seeds⁶⁾ and contrary to the galactanase (F-III) from *Rhizopus niveus*,⁸⁾ which hydrolyzed native coffee bean arabinogalactan, liberating arabinose, galactose and galactobiose. The extensive branching of (1 $\rightarrow$ 6)-linked galactose chains on O-3 by arabinofuranosyl units in grape AGp⁵⁾ prevented the enzyme from acting on this polymer by steric hindrance. The purified enzyme was an endo- β -(1 $\rightarrow$ 6)-D-galactanase since it liberated a mixture of oligosaccharides, β -(1 $\rightarrow$ 6)-D-galactobiose, and to a lesser extent galactose, being greatly predominant. Although it had not been possible to characterize higher oligomers separately (up to DP 7) due to insufficient amounts, compositional and methylation structural analysis and hydrolysis by β -D-galactosidase from *Escherichia coli* of the reaction products in the admixture found that galactose was the only sugar constituent and that only β -(1 $\rightarrow$ 6) linkages occurred. So, the enzyme was specific for (1 $\rightarrow$ 6)-linked galactose chains, (1 $\rightarrow$ 3)-linked chains being unhydrolyzed, unlike the exo-galactanase from radish seeds,⁶⁾ which cleaves both types of chains. Tsumuraya *et al.*⁹⁾ reported that Smith degradation of a radish leaf AGp produced β -(1 $\rightarrow$ 6)-D-galactooligosaccharides-(1 $\rightarrow$ 1)-glycerol up to DP 4. According to the detailed study of Tsumuraya *et al.*,⁴⁾ a desarabinosylated radish root AGp would carry oligosaccharidic β -(1 $\rightarrow$ 6)-D-galactan side-chains of various DPs branched on the β -(1 $\rightarrow$ 3)-D-galactan main chain. Our study is not inconsistent with this statement, but more work is needed to estimate DPs of (1 $\rightarrow$ 6)-linked galactose chains in the grape AGp.

Work is now in progress for structural elucidation of various AGps using in sequence

our purified α -L-arabinofuranosidase and this specific endo- β -(1 $\rightarrow$ 6)-D-galactanase.

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References

- 1) G. B. Fincher, B. A. Stone and A. E. Clarke, *Annu. Rev. Plant Physiol.*, **34**, 47 (1983).
- 2) Y. Akiyama, S. Eda and K. Kato, *Agric. Biol. Chem.*, **46**, 1395 (1982).
- 3) Y. Tsumuraya, Y. Hashimoto and S. Yamamoto, *Carbohydr. Res.*, **161**, 113 (1987).
- 4) Y. Tsumuraya, N. Mochizuki, Y. Hashimoto and P. Kováč, *J. Biol. Chem.*, **265**, 7207 (1990).
- 5) L. Saulnier and J.-M. Brillouet, *Carbohydr. Res.*, **188**, 137 (1989).
- 6) M. Sekimata, K. Ogura, Y. Tsumuraya, Y. Hashimoto and S. Yamamoto, *Plant Physiol.*, **90**, 567 (1989).
- 7) Y. Hashimoto, Y. Tsujisaka and J. Fukumoto, *Nippon Nōgeikagaku Kaishi*, **43**, 831 (1969).
- 8) Y. Hashimoto, *Nippon Nōgeikagaku Kaishi*, **45**, 147 (1971).
- 9) Y. Tsumuraya, Y. Hashimoto, S. Yamamoto and N. Shibuya, *Carbohydr. Res.*, **134**, 215 (1984).
- 10) J.-M. Brillouet and B. Carré, *Phytochemistry*, **22**, 841 (1983).
- 11) R. Waibel, R. Amado and H. Neukom, *J. Chromatogr.*, **197**, 86 (1980).
- 12) J.-M. Brillouet, J.-C. Moulin and E. Agosin, *Carbohydr. Res.*, **144**, 113 (1985).
- 13) J. Melrose and R. J. Sturgeon, *Carbohydr. Res.*, **118**, 247 (1983).
- 14) S.-I. Hakomori, *J. Biochem. (Tokyo)*, **55**, 205 (1964).
- 15) P. Albersheim, D. J. Nevins, P. D. English and A. Karr, *Carbohydr. Res.*, **5**, 340 (1967).
- 16) L. Saulnier, J.-M. Brillouet and J.-P. Joseleau, *Carbohydr. Res.*, **182**, 63 (1988).
- 17) S. G. Voirin, R. L. Baumes, S. M. Bitteur, Z. Y. Günata and C. L. Bayonove, *J. Agric. Food Chem.*, **38**, 1373 (1990).
- 18) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
- 19) U. K. Laemmli, *Nature (London)*, **227**, 680 (1970).
- 20) B. R. Oakley, D. R. Kirsch and N. R. Morris, *Anal. Biochem.*, **105**, 361 (1980).
- 21) N. Nelson, *J. Biol. Chem.*, **153**, 375 (1944).