Production of Substrate for Galactose Oxidase by Depolymerization of an Arabinogalactan-Peptide from Wheat Flour

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Water extractable arabinogalactan-peptide (WE-AGP) isolated from white wheat flour was depolymerized enzymatically to liberate substrate for a galactose oxidase from *Dactylium dendroides*. A crude liquid pectolytic preparation from *Aspergillus niger* (p70) displayed activities capable of converting WE-AGP into a substrate for galactose oxidase. The most favorable substrate was observed when WE-AGP was not fully depolymerized into galactose and arabinose. α-L-Arabinofuranosidase B from *A. niger* was also able to produce substrate from WE-AGP; arabinofuranosidase-treated WE-AGP was a better substrate for galactose oxidase than galactose. Treatment by the crude p70 and purified enzymes showed that α-L-arabinofuranosidase was partly responsible for the production of substrate, whereas *β*-galactosidase did not result in any substrate production or improve the effect of α-L-arabinofuranosidase. However, the positive effect of α-L-arabinofuranosidase was increased when p70 was added at the same level of arabinofuranosidase activity, suggesting that additional enzyme activities present in p70 were responsible for production of substrate for galactose oxidase.

Keywords: *Arabinogalactan; Aspergillus niger; Dactylium dendroides; depolymerization; galactose oxidase; wheat*

INTRODUCTION

Arabinogalactan-peptide (AGP) is found in most higher plants. It is a group of water-soluble macromolecules characterized by a highly branched structure. The carbohydrate moiety of wheat flour arabinogalactan peptide (WE-AGP) consists of a (1→3)-*β*-D-galactan backbone with branches of (1→6)-*β* linked D-galactosyl residues, heavily substituted by α-L-arabinose units (Fincher and Stone, 1974; Pellerin et al., 1993). The amino acid composition of the peptidic part of WE-AGP is unusual as it contains a large quantity of 4-hydroxyproline. This amino acid might serve as a link to the carbohydrate part by glycosidic attachment to the galactan chain (Fincher et al., 1974; Neukom, 1976; McNamara and Stone, 1981; Strahm et al., 1981).

In white wheat flours, WE-AGP occur as minor components (0.2−0.4% w/w) (Loosveld et al., 1997). It commonly contains less than 10% protein and the weight ratio between arabinose and galactose is ∼0.7 (Izydorczyk et al., 1991).

Several enzymes are probably required to obtain a complete depolymerization of the arabinogalactan part of WE-AGP: α-L-arabinofuranosidase releasing arabinose from the galactan chain, galactanases hydrolyzing (1→3)-*β*- and -(1→6)-*β*-linked D-galactose residues, and *β*-galactosidase liberating single galactose units. Some authors have successfully hydrolyzed grape and wine arabinogalactan peptides with purified α-L-arabinofuranosidase (Saulnier et al., 1992), (1→6)-*β*-D-galactanase (Brillouet et al., 1991) and *exo*-(1→3)-*β*-D-galactanase (Pellerin and Brillouet, 1994).

WE-AGP has not been reported to exhibit marked functional properties during the breadmaking of wheat flours unlike arabinoxylans, for example. The objective of the present work was to explore the possibility to use WE-AGP or degradation products of WE-AGP as a substrate for an oxidative enzyme, namely galactose oxidase. Indeed, the use of oxidative enzymes in breadmaking has received increasing attention because a number of chemical oxidants such as potassium bromate has been banned. The most frequently used oxidative enzyme for bread production is glucose oxidase. However, it has some limitations because it depends on the existence of glucose in a dough. Normally glucose is available in the beginning of the breadmaking process but is fast used by yeasts. Therefore the oxidative effect is strong only during dough mixing. The purpose of this work was to find another oxidation system which relies on a carbohydrate substrate naturally occurring in the flour.

Galactose oxidase (EC 1.1.3.9) is a copper-containing enzyme secreted by certain filamentous fungi. This enzyme catalyzes the oxidation of a range of primary alcohols, including the C-6 position of D-galactose and the terminal D-galactose of oligosaccharides, to the corresponding aldehydes. Galactose oxidase does not oxidize either D-glucose or L-galactose at all, even though the two isomers of galactose both have a primary alcohol group at the C-6 position (Avigad et al., 1962). Oxidation is accompanied by a reduction of molecular oxygen and the release of hydrogen peroxide (Knowles and Ito, 1993).

Galactose oxidase is very resistant to denaturing effects of heat, pH, and protease treatment (Mendonca

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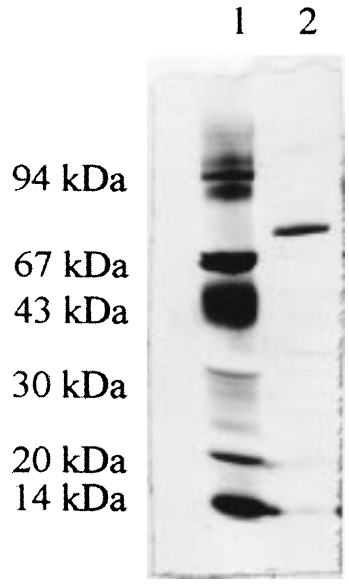

Figure 1. SDS−PAGE (8−16% TG) analysis of purified galactose oxidase as revealed by silver staining: lane 1, LMW electrophoresis calibration proteins (Pharmacia Biotech), and lane 2, purified galactose oxidase.

et al., 1988). It has been reported that galactose oxidase acts on some oligosaccharides and polysaccharides, with D-galactose in a terminal nonreducing position, substantially faster than on galactose (Avigad et al., 1962; Bretting et al., 1987). The tetrasaccharide stachyose is oxidized 6 times more rapidly than galactose, and guaran, a high molecular weight mannan containing terminal galactosyl units as branches, is oxidized twice as fast as galactose (Avigad et al., 1962).

The stability of this enzyme combined with its broad substrate specificity with respect to saccharides makes an attractive possibility as an alternative oxidative enzyme for use in breadmaking. In the present work, we describe the depolymerization of WE-AGP and its conversion into a useful substrate for galactose oxidase by enzyme treatment.

MATERIALS AND METHODS

Enzymes. A commercial crude ferment of galactose oxidase from *Dactylinum dendroides*, obtained from Sigma (St. Louis, MO), was purified to ensure that any oxidative effect obtained was solely a result of galactose oxidase activity.

The preparation (60 mL) was purified by desalting the crude ferment on a 550-mL Sephadex G25 C column (XK50/28, Pharmacia Biotech) equilibrated in 20 mM triethanolamine (TEA), pH 7.3 at a flow rate of 15 mL/min. The desalted sample (150 mL) was then applied to a 20 mL Source Q (30 μm) column (XK 16/10, Pharmacia Biotech) equilibrated in 20 mM TEA buffer at a flow rate of 5 mL/min. The nonbound fraction was then collected (180 mL). $(NH_4)_2SO_4$ was added to the sample to a final concentration of 1.0 M. The sample was then loaded on a 20-mL Source HIC column (phenyl, XK 16/10, Pharmacia Biotech) equilibrated in 20 mM NaAc buffer, pH 5.0 + 1.0 M $(NH_4)_2SO_4$ (buffer A). After the column was washed with 50 mL of buffer A, the galactose oxidase was eluted with a 200-mL linear gradient from 1.0 to 0 M $(NH_4)_2SO_4$ in buffer A at a flow rate of 5 mL/min, during which 7.5-mL fractions were collected. The eluted protein was subjected to SDS−PAGE using a Novex system with precast gels (Novex, San Diego, CA) and presented a single band after silver staining performed according to the manufacturer (Figure 1). An approximate molecular weight of 69 kDa was estimated for the purified galactose oxidase by comparison with a LMW protein calibration kit for electrophoresis (Pharmacia Biotech).

A crude liquid enzyme preparation (p70) from *Aspergillus niger* was obtained from Danisco Ingredients (Brabrand, Denmark). Horseradish peroxidase (P-81259) and β-galactosidase from *A. niger* (G-3522) were purchased from Sigma and α-L-arabinofuranosidase B from *A. niger* was obtained from Megazyme (Wicklow, Ireland).

Units applied for α-L-arabinofuranosidase and horseradish peroxidase and β-galactosidase were in accordance with the definition provided by the manufacturer. One unit of horseradish peroxidase produced 1 mg of purpurogallin from pyrogallol in 20 s at pH 6.0 at 20 °C. One unit of β-galactosidase hydrolyzed 1.0 μmol of *o*-nitrophenyl β-D-galactoside to *o*-nitrophenol and D-galactose per min at pH 4.0 at 25 °C and one unit of α-L-arabinofuranosidase hydrolyzed 1.0 μmol *p*-nitrophenyl α-L-arabinofuranoside (pNPA) per min at pH 4.0 at 40 °C.

One unit of galactose oxidase was defined as the amount of enzyme that produced 1 nmol of hydrogen peroxide per second with galactose as substrate at room temperature at pH 5.75.

Enzyme Screening. The p70 enzyme solution was screened for different activities able to depolymerize WE-AGP. *p*-Nitrophenyl substrates, *p*-nitrophenyl β-D-galactoside (pNPG), *p*-nitrophenyl 6-*O*-β-D-galactopyranosyl-β-D-galactopyranoside (pNPGG) and pNPA, all from Sigma, were used to detect β-D-galactosidase, (1→6)-β-D-galactanase and α-L-arabinofuranosidase, respectively. Fifty microliters of p70 solution was added to 0.5 mL of 0.5 mM pNPA, pNPG, or pNPGG dissolved in 50 mM Na−acetate buffer, pH 5.0. Incubation took place for 10 min at room temperature. One milliliter of 1 M Na_2CO_3 was added, and the yellow color of released pNP was detected at 410 nm in a spectrophotometer. Activities were calculated using a molar extinction coefficient of 13 700 M^{-1} cm^{-1}.

ABTS Assay for Galactose Oxidase. An ABTS reagent was prepared by dissolving 6 mg of 2,2-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Sigma) in 10 mL of 0.1 M phosphate buffer, pH 6.4, together with 50 U of galactose oxidase and 20 U of peroxidase. Na−phosphate buffer, 0.1 M, pH 6.4, was added to obtain a total volume of 25 mL.

Eight hundred microliters of ABTS reagent was incubated with 50 μL of substrate in cuvettes for 30 min at room temperature. The absorbance was immediately read at 420 nm.

Enzyme-treated WE-AGP, galactobiose (6-*O*-β-D-galactopyranosyl-D-galactose) (Sigma), or galactose (Sigma) was used as substrate.

Isolation of WE-AGP. Twenty grams of water extractable pentosan (WSP) from Thesee flour (Faurot et al., 1995) was dissolved in 800 mL of water. The solution was adjusted using 96% ethanol to obtain a final concentration of 60% (v/v) ethanol, and the mixture was held overnight. Arabinoxylan was removed by centrifugation (12000*g* for 30 min at 4 °C).

The supernatant was filtered through 0.45-μm filters (Whatman, Maidstone, England), adjusted to 80% (v/v) ethanol, and held for 2 h at 4 °C to precipitate WE-AGP. The solution was then centrifuged at 12000*g* for 30 min at 4 °C, and the precipitate was redissolved in 100 mL water and later freeze-dried. The carbohydrate content was analyzed by gas liquid chromatography on a DB-225 capillary column of their alditol acetates obtained after sulfuric acid hydrolysis of the WE-AGP-samples; inositol was used as internal standard (Blakeney et al., 1983). The protein content was determined according to the Dumas method (AOAC, 1990; Bicsak, 1993).

Depolymerization of WE-AGP. Enzyme reactions with WE-AGP were performed at room temperature in 0.05 M Na−acetate buffer, pH 5.0. The enzyme reactions were stopped by boiling for 5 min. Samples were centrifuged at 12000*g* for 10 min, and the supernatant was used for further studies. All experiments were performed in duplicate, details are given in figure captions.

Oxidation of WE-AGP. Hydrolysis of WE-AGP was followed concomitantly with oxidation by galactose oxidase in a spectrophotometer using 1.8 mL of ABTS reagent (pH 5.75) incubated with 50 μL of enzyme solution and 100 μL of WE-AGP solution (20 mg/mL). The reaction was followed kinetically for 45 min at 420 nm. Respectively, α-L-arabinofuranosi-

Table 1. Composition of Water-Soluble Pentosans (WSP) from White Wheat Flour and Water-Extractable Arabinogalactan-Peptide (WE-AGP) Extracted from WSP and Yields of the Extraction

	WSP[a] (% dry basis)	WE-AGP (% dry basis)	yield (%)
protein	42.9	37.2	23.0
arabinose	19.4	17.6	23.7
xylose	21.9	1.9	2.3
galactose	9.9	22.8	60.1
glucose	3.5	0.7	4.3

[a] According to Faurot et al., 1995.

dase (1.5 U), β-galactosidase (4 mU), and p70 (7 μL) were applied as hydrolyzing enzymes.

Gel Permeation Chromatography. Enzyme-treated WE-AGP was filtered through 0.45-μm filters (Whatman) and subjected to gel permeation chromatography using a Waters system with refractometric detection. One hundred microliters was injected on a Superdex 75 HR 10/30 column (Pharmacia, separation range 3000−70000 for globular proteins) at a flow rate of 1 mL/min in 0.1 M Na−acetate buffer, pH 5.0.

HPLC. Enzyme-treated WE-AGP was centrifuged (14000*g*, 15 min), filtered (0.45-μm filters, Whatman), and subjected to HPLC, using a Waters system with refractometric detection.

A 5-μL sample was injected on a Polyspher CHCA column (Merck, Darmstadt, Germany) in which polysaccharides and monosaccharides were separated at 85 °C with water as the mobile phase (flow rate 0.5 mL/min). With the use of standard curves, galactose and arabinose were quantified, whereas galactobiose was not quantified but expressed according to peak area.

RESULTS

Composition of WE-AGP. The carbohydrate and protein contents of isolated WE-AGP were analyzed and compared to the starting material, WSP (Table 1). WSP contained mainly arabinoxylan and arabinogalactan-peptide (Faurot et al., 1995).

High yields of galactose and protein were recovered in the isolated WE-AGP fraction, whereas only a part of the total amount of arabinose was found. This corresponded to the distribution of arabinose between arabinoxylan and arabinogalactan. Only very low amounts of xylose were observed in the isolated WE-AGP, suggesting a good separation from arabinoxylan. A total of 5.25 g of WE-AGP was purified from 20 g of Thesee WSP, giving a total yield of 26.25%.

Depolymerization of WE-AGP. Depolymerization of WE-AGP using a crude liquid enzyme preparation from *A. niger* (p70) was followed by gel permeation chromatography (Figure 2). A shift toward low molecular weight molecules was observed, when WE-AGP had been incubated with p70. After 18 h of reaction time, almost all high molecular compounds had degraded into small molecules or simple sugars, suggesting that p70 contains enzyme activities capable of depolymerizing WE-AGP.

HPLC was used to identify the released sugar components from WE-AGP (Figure 3). Incubation with p70 resulted in the release of galactobiose, galactose, and arabinose. Two unidentified components were detected in small amounts (eluted after 8 and 11 min).

The time course of the sugar release was followed and quantified. Arabinose was released at a high rate and reached a maximum at 5 h of incubation time, whereas galactose was released continuously up to 17 h (Figure 4). Galactobiose was produced at the beginning of the incubation period, reaching a maximum at 7 h, but was hydrolyzed into galactose later during incubation. Eighty-three percent of the total amount of arabinose from WE-AGP was released, whereas 24% of the galactose was released as free galactose. A fraction of approximately 4% of the WE-AGP (determined by area) was resistant to degradation by p70.

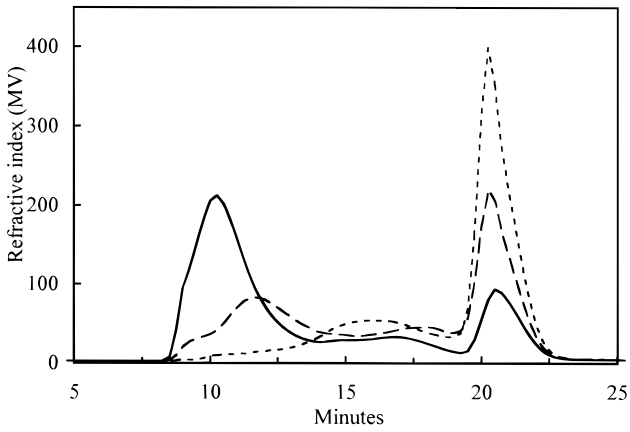

Figure 2. GPC (Superdex 75 HR 10/30, Pharmacia) profiles of WE-AGP incubated with p70 for 30 min (— —) and 18 h (- - -) of reaction time and WE-AGP exposed for 18 h to inactivated p70 (—). Reaction mix: 0.5 mL WE-AGP, 20 mg/mL, and 10 μL p70.

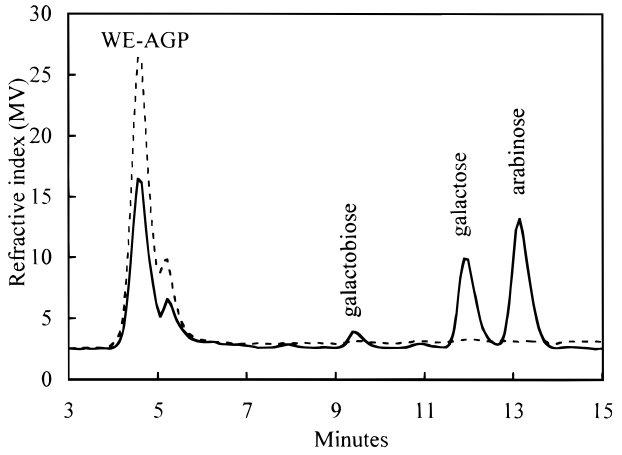

Figure 3. HPLC (Polyspher CHCA, Merck) elution profiles of WE-AGP incubated with p70 (—) and WE-AGP exposed to inactivated p70 (- - -). Reaction mix: 5 mL WE-AGP, 7.5 mg/mL, and 30 μL p70, 7 h.

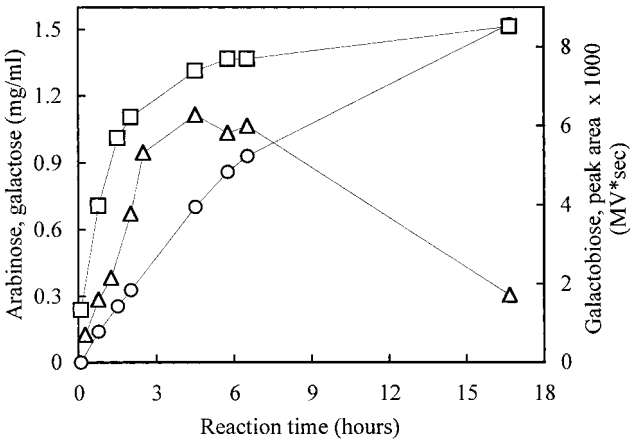

Figure 4. The release of galactose (○), arabinose (□), and galactobiose (△) from WE-AGP during incubation with p70 quantified by HPLC (Polyspher CHCA, Merck). Reaction mix: 5 mL WE-AGP, 7.5 mg/mL, and 30 μL p70.

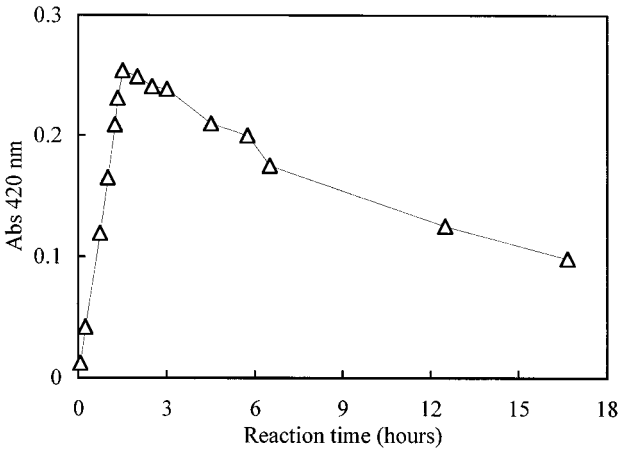

Figure 5. Production of substrate for galactose oxidase during the incubation of WE-AGP with p70, determined by ABTS assay. Reaction mix: 5 mL WE-AGP, 7.5 mg/mL, and 30 μL p70.

Oxidation of Depolymerization Products from WE-AGP by Galactose Oxidase. The formation of substrate for galactose oxidase from WE-AGP was followed indirectly by oxidation of ABTS. This method makes it possible to quantify spectrophotometrically the available oxidizable substrate for galactose oxidase. The absorbance of oxidized ABTS is a function of the amount of substrate available for galactose oxidase.

WE-AGP was incubated with p70, and the enzyme reaction was stopped at different time intervals to determine the course of substrate production by the ABTS assay.

The most favorable substrate for galactose oxidase was produced at the beginning of the incubation period, observed by a high oxidation rate (Figure 5). Further incubation with p70 resulted in products from WE-AGP which were less favorable for oxidation by galactose oxidase.

Maximum oxidation corresponded to the time when most of the arabinose was released, when only a minimum of galactose was produced and a relatively large amount of galactobiose was still present (Figure 4). Thus, maximum oxidation by galactose oxidase occurred before WE-AGP was fully depolymerized.

Three different galactose containing compounds were compared as substrates for galactose oxidase using the ABTS assay: WE-AGP extensively treated with α-L-arabinofuranosidase, galactose, and galactobiose (Figure 6). Arabinose-free WE-AGP was the best substrate of the three, when compared on a galactose equivalent basis. Galactose was approximately one-third as reactive, while galactobiose resulted in the lowest oxidation of the three. However, by comparing galactose and galactobiose on a molar basis, galactobiose was clearly more favorable as a substrate than galactose itself.

Effect of Selected Enzymatic Activities on Oxidation. Enzyme reactions on WE-AGP were performed using either a pure α-L-arabinofuranosidase B from *Aspergillus niger* or a p70 solution at the same level of arabinofuranosidase activity (measured using pNPA). The depolymerization of WE-AGP and subsequent oxidation by galactose oxidase were followed directly over time by a spectrophotometer using the ABTS assay (Figure 7).

The addition of α-L-arabinofuranosidase alone resulted in a substrate that could be oxidized, but the oxidation was increased when p70 was added. The initial oxidation rate was approximately 3 times higher when p70 was applied.

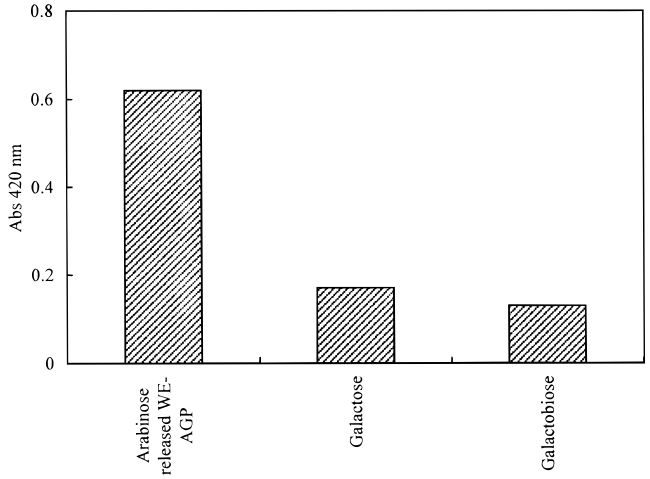

Figure 6. Oxidation of different galactose compounds by galactose oxidase, compared on galactose equivalent basis. Twenty-five milliliters of WE-AGP treated with α-L-arabinofuranosidase (10 U/mL, 3 h), galactobiose, and galactose (at 50 mM "galactose"), and 975 μL ABTS reagent, 10 min.

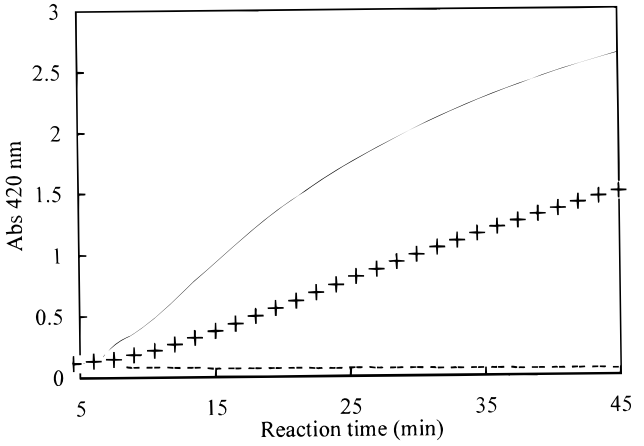

Figure 7. Oxidation of WE-AGP by galactose oxidase during incubation with a pure α-L-arabinofuranosidase from *Aspergillus niger* (+) or the p70 solution (—), determined by the ABTS assay. Control: WE-AGP incubated with galactose oxidase (- -).

In addition to α-L-arabinofuranosidase, β-galactosidase, and (1→6)-β-D-galactanase activities were also detected in the p70 enzyme solution using pNPG and pNPGG. α-L-Arabinofuranosidase and β-galactosidase were incubated individually or in combination with WE-AGP, and the product was evaluated as a substrate for galactose oxidase (Figure 8). Oxidation was only observed when WE-AGP was treated with α-L-arabinofuranosidase. The addition of β-galactosidase did not improve oxidation. This corresponds to the fact that this enzyme was not able to hydrolyze WE-AGP without the release of arabinose (Table 2).

No synergistic effect on oxidation was observed when both enzymes were combined. Thus, additional activities to α-L-arabinofuranosidase are responsible for the release of oxidizable substrate for galactose oxidase in the p70 solution, and they must originate from activities different from β-galactosidase. Previous experiments showed that oxidation of ABTS was inhibited by compounds contained in p70 (data not shown), suggesting that the positive effects of p70 are actually more

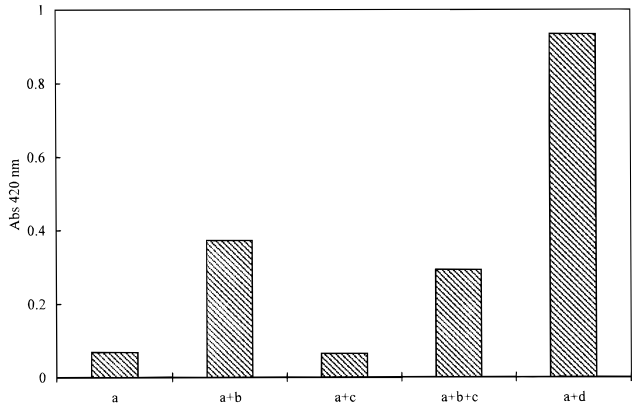

Figure 8. Oxidation of WE-AGP by (a) galactose oxidase alone and (b) after 15 min of incubation with α-L-arabinofuranosidase, (c) β-galactosidase, and (d) the p70 solution determined by the ABTS assay.

Table 2. Released Sugars from WE-AGP When Incubated with Hydrolyzing Enzymes

	released saccharides		
WE-AGP incubated with	arabinose	galactose	galactobiose
α-L-arabinofuranosidase	+	−	−
β-galactosidase	−	−	−
α-L-arabinofuranosidase, β-galactosidase	+	(+)	−
p70	+	+	+

profound. This inhibitor was a small molecule, not of protein nature and not an agent chelating cupper.

DISCUSSION

Galactose oxidase is, according to the literature, able to act on some oligosaccharides with galactose in a terminal nonreducing position substantially faster than on free galactose (Bretting et al., 1986). These investigations into the structural requirements for oligosaccharides from snail galactan to bind to the galactose oxidase from *Dactylium dendroides* indicated that galactose oxidase recognizes a determinant comprising more than one galactose residue. It was found that (1→6)-β-linked D-galactose residues were oxidized substantially faster than the corresponding (1→3)-β-D-linked oligosaccharide. Furthermore, the (1→3)-β- or (1→6)-β-linked D-galactose has to be in the terminal position to be accessible to galactose oxidase. A branched tetrasaccharide, exposing two terminal galactose residues in a (1→3)-β- and (1→6)-β-linkage, was the most complementary structure in the native galactan to associate with galactose oxidase. This compound was oxidized 10 times faster than galactose.

The treatment of WE-AGP with α-L-arabinofuranosidase could make (1→6)-β-linked D-galactose residues accessible for galactose oxidase, corresponding to the fact that α-L-arabinofuranosidase-treated WE-AGP is a better substrate compared to galactose. The enhanced effect from p70 in liberating substrate for galactose oxidase is still unclear. Several enzymes could work synergistically, modifying the arabinogalactan in such a way that the favorable structural requirement for galactose oxidase is obtained − for example, protease liberating arabinogalactan from the peptidic part, (1→6) and (1→3)-β-D-galactanases cleaving the galactan backbone, and finally α-L-arabinofuranosidase liberating arabinose in a way that branched terminal tetrasaccharides are exposed.

CONCLUSION

A crude liquid enzyme preparation from *Aspergillus niger* (p70) displayed activities capable of depolymerizing WE-AGP. Three different released sugars components were identified: galactobiose, galactose, and arabinose.

The formation of substrate for galactose oxidase from WE-AGP was followed indirectly by oxidation of ABTS and showed that the most favorable substrate for galactose oxidase was present when WE-AGP was not fully depolymerized by p70.

α-L-Arabinofuranosidase B from *A. niger* was also able to produce substrate from WE-AGP. WE-AGP treated with α-L-arabinofuranosidase was approximately three times more efficient than galactose as a substrate.

Treatment by different enzymes found in p70 showed that α-L-arabinofuranosidase was partly responsible for the production of substrate, whereas β-galactosidase did not result in any production or did not improve the effect observed from α-L-arabinofuranosidase. However, the effect observed when WE-AGP was incubated with α-L-arabinofuranosidase was increased when p70 was added, at the same level of arabinofuranosidase activity, suggesting that alternative enzyme activities were responsible for the production of substrate for galactose oxidase. These activities remain to be found and isolated to reveal the exact contribution of the p70 enzyme solution to the production of substrate from WE-AGP.

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