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# Structure of Plant Cell Walls

## IX. PURIFICATION AND PARTIAL CHARACTERIZATION OF A WALL-DEGRADING ENDO-ARABANASE AND AN ARABINOSIDASE FROM *BACILLUS SUBTILIS*[1]

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### ABSTRACT

**Wild type *Bacillus subtilis*, when grown on beet araban, secretes into its culture medium an endo-arabanase and two arabinosidases. An alternate procedure to one previously described (Kaji A, T Saheki 1975 Biochim Biophys Acta 410: 354–360) has been developed for the purification of the endo-arabanase. The purified endo-arabanase is shown to be homogeneous by sodium dodecyl sulfate-urea disc gel electrophoresis (molecular weight ≃ 32,000) and by isoelectric focusing (pI = 9.3). The endo-arabanase, acting on a branched araban substrate, has maximal activity at pH 6.0 and preferentially cleaves 5-linked arabinosyl residues. One of the arabinosidases (molecular weight ≃ 65,000, pI = 5.3) has been purified to the point that it contains only one quantitatively minor contaminant, as shown by sodium dodecyl sulfate-urea disc gel electrophoresis and isoelectric focusing. The purified arabinosidase, acting on *p*-nitrophenyl-α-L-arabinofuranoside, has maximal activity at pH 6.5, and, when acting on a branched araban substrate, preferentially attacks nonreducing terminal arabinosyl residues linked to the 2 or 3 position of other arabinosyl residues. Neither of the two purified enzymes is capable of hydrolyzing a variety of carbohydrate substrates which lack arabinosidic linkages. The purified endo-arabinase is shown to be capable of releasing arabinosyl oligomers from the walls of suspension-cultured sycamore cells, thereby suggesting its usefulness as a probe in studying the structure of the araban component of primary cell walls.**

The major polysaccharide components of the primary cell walls of dicots, as well as some general features of the interconnections of the wall polysaccharides, have been described (3, 14, 24). Our present level of understanding of the structure of primary cell walls has been achieved, in part, by the use of highly purified enzymes capable of selective cleavage of the wall polysaccharides. The advantage in using such enzymes lies in their ability to degrade the complex polymers present in the wall to a limited set of identifiable oligomers. Classical acidic or basic extraction procedures are not suitable because they result in the simultaneous cleavage of a number of different types of bonds present in the cell wall (1). This results in the solubilization of a heterogeneous mixture of oligo- and polysaccharides that are too complex to piece together into a workable model of primary cell wall structure.

Structural information concerning the interconnection of the wall polysaccharides has been deduced from analysis of the oligosaccharide products produced by the purified polysaccharide-degrading enzymes. Included among the oligosaccharide products were fragments containing pieces of two or more of the wall polysaccharides (24). These findings suggested that an important cross-linking polymer was composed of araban and galactan polysaccharides attached to a rhamnogalacturonan backbone (24). The detailed structures of the araban and galactan have not been determined. Primarily, this has been due to the unavailability of purified enzymes capable of degrading these polysaccharides to identifiable oligomers.

Labavitch *et al.* (15) have recently described the isolation and purification of a β-1,4-galactanase from *Bacillus subtilis* that degrades a structural component of primary cell walls. Partial characterization of the products of galactanase digestion of suspension-cultured sycamore cell walls indicates that the majority of the arabinosyl residues released by galactanase action are present as polymers of degree of polymerization of at least 10. This contradicts previous suggestions (19) that the wall araban-galactan exists as a 1,4-linked galactan backbone with arabinosyl mono- and disaccharide substituents present on the backbone. The presence of arabinosyl residues as araban polymers in the galactanase-solubilized polysaccharides suggests that arabanases would prove valuable in the further elucidation of primary cell wall structure.

Recently, Kaji and Saheki (12) reported the isolation and purification of an endo-arabanase from *B. subtilis* that shows promise as an enzyme capable of assisting the previously purified galactanase in the elucidation of the structure of the primary cell wall polysaccharides. The purification procedure employed by Kaji and Saheki makes use of alternating hydroxylapatite and gel exclusion chromatography resulting in a relatively low yield of endo-arabanase. The present paper describes the isolation and purification of this endo-arabanase via an alternate procedure leading to much higher yields of purified enzyme. The physical properties of the endo-arabanase have been further elucidated. The purification procedure described here also allows for the isolation and purification of an exo-arabanase, *i.e.* an arabinosidase. Aside from possessing different modes of attack on an araban substrate, the two enzymes isolated are also shown to possess different linkage specificity with respect to their hydrolysis of a highly branched araban. Evidence is presented indicating that these enzymes will be useful in the structural analysis of primary cell wall polysaccharides.

## EXPERIMENTAL PROCEDURE

### MATERIALS

Diethylaminoethyl (DEAE)-Sephadex A-50 and carboxymethyl (CM)-Sephadex C-50 were purchased from the Sigma Chemical Co. Hydroxylapatite (HTP grade), agarose 1.5m (100–200 mesh), and Bio-Gel P-2 (200–400 mesh) were purchased from Bio-Rad. The *p*-nitrophenyl derivatives of α- and β-D-xylopyranoside, α- and β-D-glucopyranoside, and β-D-fucopyranoside were obtained from Koch-Light; *p*-nitrophenyl-β-D-galactopyranoside was purchased from Sigma; *p*-nitrophenyl-α-L-arabinofuranoside was syn-

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thesized by the procedure of Fielding and Hough (7) and was shown to have the same physical and chemical properties as originally reported. Other substrates used included carboxymethylcellulose (type 7MP), purchased from Hercules Powder, citrus polygalacturonic acid, a gift from Sunkist Growers, and larch galactan, obtained from Koch-Light. Citrus pectic galactan was purified as previously described (15). All other reagents used were analytical grade, or as indicated.

### PURIFICATION OF POLYSACCHARIDE SUBSTRATES

**Araban.** "Pure" beet araban was obtained from Koch-Light (Lot 52568). Neutral sugar analysis (2) indicated that the araban was of approximately 66% arabinose by weight. This araban substrate was further purified by the following procedure. The araban (10 g) was dissolved in 500 ml 0.5 N NaOH containing 8.0 g $NaBH_4$ and was refluxed at 95 C for 18 hr. The resulting suspension was cooled to room temperature and the pH slowly adjusted to 5.0 with glacial acetic acid. Residual solids were removed by centrifugation. The resulting supernatant solution was dialyzed against 10 mM K-phosphate (pH 7.0) and applied to a DEAE-Sephadex A-50 column (2.5 × 21 cm) previously equilibrated with 10 mM K-phosphate (pH 7.0). The araban was washed through the column with 250 ml of the phosphate buffer. The void and wash fractions were pooled, exhaustively dialyzed against deionized $H_2O$, and lyophilized to dryness, giving 2.5 g of a white powder. This purified araban is approximately 94% arabinose by weight (2). Residual impurities are primarily galactose (3%) and glucose (2%). Methylation analysis (Table I) (8, 24) indicated that the araban is a highly branched polysaccharide. The purified araban voids a Bio-Gel P-2 column (1.4 × 113 cm) equilibrated and run in $H_2O$ at 50 C.

**Debranched Araban.** The highly branched araban described above was converted to a partially "debranched araban" by the following procedure. The purified araban (60 mg) was dissolved in 3.0 ml 10 mM Na-phosphate (pH 6.5). The araban was subjected, for 48 hr at room temperature, to the action of 0.84 units purified *B. subtilis* arabinosidase II (see under "Results and Discussion"). Toluene (5 μl) was introduced as a bacteriostat. The reaction was terminated by placing the mixture in a boiling water bath for 15 min. The resulting solution was applied directly to a Bio-Gel P-2 column (1.4 × 113 cm) which had been equilibrated with, and which was eluted with, distilled $H_2O$ at 50 C. Two orcinol-positive (4) peaks eluted from the column, one at the void volume of the column and the other at the included volume. The orcinol-positive, totally included peak was shown by neutral sugar analysis (2) to contain only monomeric arabinose. The void fractions, comprising approximately 65% of the orcinol-positive starting material, were combined, exhaustively dialyzed against $H_2O$, and lyophilized to dryness. Methylation analysis (8, 24) of the arabinosidase-treated araban (Table I) showed that this product possesses a smaller proportion of branched arabinosyl residues than the starting material. The arabinosidase-treated araban is termed "debranched araban."

Table I. Linkage analysis of araban and debranched araban substrates.

Debranched araban was generated by exhaustive treatment of araban with B. subtilis arabinosidase II followed by chromatography on Bio-Gel P-2 and dialysis against $H_2O$ (see text). Due to these purification steps, quantitative comparison of the glycosyl linkage compositions of the araban and debranched araban is not possible. Methylation analyses were performed as described (8, 24). Polysaccharides (1.0 mg) were permethylated and then hydrolyzed in 1.0 ml of 2N trifluoroacetic acid containing 0.10 mg of myo-inositol. The results are expressed in terms of the arabinosyl linkages rather than as their methyl derivatives (24). For example, 5-linked arabinosyl residues represent the assayed amount of the partially methylated alditol acetate 2,3-di-O-methyl-1,4,5-tri-O-acetyl arabinitol.

| Arabinosyl Residue | Arabinosyl Residues Recovered | |
|---|---|---|
| | Araban | Debranched Araban |
| | mole % | |
| Terminal | 25.7 | 13.0 |
| 2 | 2.2 | 1.8 |
| 3 | 7.4 | 9.3 |
| 5 | 31.1 | 55.1 |
| 2,3 | 5.4 | 1.9 |
| 2,5 | 7.4 | 5.7 |
| 3,5 | 12.8 | 8.8 |
| 2,3,5 | 7.9 | 4.4 |

### METHODS

**Enzyme Assays.** Unless otherwise indicated, arabanase activity was determined by addition of 10 μl enzyme solution to 0.49 ml of a 2% solution of purified araban in 10 mM Na-phosphate (pH 6.0). After incubation for up to 1 hr at 28.5 C, the reaction mixture was terminated by the addition of the alkaline $CuSO_4$-containing Nelson-Somogyi reagent and immediate determination of reducing-sugar content (20, 22). A unit of arabanase activity is defined as the amount of enzyme which liberates 1 μmol of arabinose-reducing equivalents ($\Delta A_{520\ nm}$ = 0.45)/min under the above conditions.

Arabinosidase activity was measured by addition of 5 μl enzyme solution to 0.495 ml of a 2% solution of *p*-nitrophenyl-α-L-arabinofuranoside in 10 mM Na-phosphate (pH 6.5). After incubation at 28.5 C for various times, the reactions were terminated by the addition of 1.0 ml of a solution of 2 mM EDTA in 2 M $NH_4OH$ (5). The amount of *p*-nitrophenol liberated was measured at 400 nm. An arabinosidase unit is defined as the amount of enzyme which liberates 0.1 μmol *p*-nitrophenol ($\Delta A_{400\ nm}$ = 1.7)/min.

**Neutral Sugar and Glycosyl Linkage Analysis.** Neutral sugar compositions were determined as previously described (2). Glycosyl linkage analyses were performed on methylated (8) samples with analysis of the derived partially methylated alditol acetates as described (24) except that the partially methylated aldoses were reduced with sodium borodeuteride rather than with sodium borohydride to aid in mass spectral analysis of otherwise symmetrical derivatives (15). The partially methylated alditol acetates were identified by both gas chromatographic retention times and mass spectral analysis of the column effluent. MS was performed on a Hewlett-Packard model 5928A mass spectrometer controlled by a model 5933A data system. Quantitation of the alditol acetates as well as of the partially methylated alditol acetates was carried out by computer-assisted integration of the Hewlett-Packard model 7620A gas chromatographic flame ionization detector response (unpublished procedure of H. Albert and P. Albersheim) and the integration values were divided by the calculated response values for each partially methylated alditol acetate derivative (23).

**Additional Assays.** Reducing sugars were determined by the method of Nelson (20) as modified by Somogyi (22). Arabinose concentrations were determined by the orcinol assay (4). Protein was determined either by differential absorbance at 280 and 260 nm (17) or by the procedure of Lowry *et al.* (18) using BSA as a standard.

The zymogen technique of Eriksson and Pettersson (6), using purified araban as substrate, was used to locate arabanase and arabinosidase activities in the isoelectric focusing gels.

**Mol Wt Determinations.** Mol wt of purified enzymes were estimated by gel filtration chromatography and/or by polyacrylamide disc gel electrophoresis. Gel filtration chromatography was carried out on an agarose 1.5m column (1.5 × 70 cm) equilibrated with and run in 10 mM Na-phosphate (pH 6.8). The following standards (from Sigma) were used for calibration of the column: blue dextran (mol wt about 2 × $10^6$), bovine thyroglobulin (mol wt 670,000), BSA (mol wt 68,000), human hemoglobin (mol wt

64,500), ovalbumin (mol wt 45,000), and carbonic anhydrase (mol wt 30,000).

Discontinous SDS-7 M urea polyacrylamide gels (10%) were prepared and run as described by Reid and Bielski (21) using the buffer system described by Laemmli (16). Thin gels (0.8 mm) were run to enhance resolution. After electrophoresis, the gels were stained for protein with Coomassie brilliant blue 250 (Sigma). Estimates of protein mol wt were made by comparison with the distance migrated by the "standard" proteins. Proteins in the standard (from Sigma) included BSA, carbonic anhydrase, ovalbumin, and bovine pancreatic ribonuclease-A (mol wt 14,000).

**Isoelectric focusing.** Isoelectric focusing was performed on an LKB-multiphor system according to the manufacturers instructions. Premade LKB-Ampholine PAG plates (range pH 3.5–9.5) were used. The pH gradient formed upon isoelectric focusing was determined using a Beckman research pH meter equipped with an Ingold surface pH electrode.

### GROWTH OF BACTERIA AND PREPARATION OF CULTURE FILTRATE

Wild type *B. subtilis* F-11 (a gift from A. Kaji, Dept. of Agricultural Chemistry, Kagawa University, Kagawa, Japan) was cultured in a beet extract medium previously described (12, 13). Beet medium (250 ml) was inoculated from a slant of the bacteria and incubated for 24 hr at 25 C on a gyratory shaker at 100 to 120 rpm. The resulting bacterial suspension was then added directly to 7.75 liters of beet medium and allowed to incubate for 90 hr at 28 C in a VirTis model 40-300AR fermentor ($A_{650\ nm}$ = 5.94 at harvest). Cells and solid debris were removed by centrifugation at 15,000*g* followed by filtration through Whatman GF/C. The culture filtrate obtained was kept at 4 C and used as the source of crude endo-arabanase and arabinosidase II.

## RESULTS AND DISCUSSION

The isolation and purification of an endo-arabanase produced by *B. subtilis* has been described previously (12). The procedure that was employed (12) in the purification of the endo-arabanase entailed the repeated alternated use of hydroxylapatite and gel filtration chromatography, leading eventually to an apparently homogeneous enzyme. The anomalous approach used by the authors in their purification of the endo-arabanase, and the low yields obtained (5.3%), induced us to try to purify this enzyme, employing a more classical approach.

### PURIFICATION OF ENDO-ARABANASE

All steps in the purification procedure subsequent to the isolation of the culture filtrate were performed at 4 C.

**Step 1. Ammonium Sulfate Precipitation.** Ammonium sulfate (3,430 g) was slowly added to 7.25 liters of culture filtrate to achieve approximately 90% saturation. Following incubation at 4 C overnight, the resulting precipitate was collected by filtration on Whatman GF/C and redissolved in 1 liter of distilled $H_2O$. The solution obtained was dialyzed first against four 12-hr changes of 10 liters of water followed by dialysis against four 12-hr changes of 10 liters of 10 mM Na-phosphate (pH 6.0).

**Step 2. CM-Sephadex C-50 Chromatography.** The dialyzed solution obtained in Step 1 was applied to a CM-Sephadex C-50 column (2.5 × 27 cm) previously equilibrated in 10 mM Na-phosphate (pH 6.0). The column was washed with 250 ml of 10 mM Na-phosphate (pH 6.0), followed by two sequential linear gradients: 0 to 100 mM NaCl in 10 mM Na-phosphate (pH 6.0) (total gradient volume 500 ml), and 100 to 500 mM NaCl in 10 mM Na-phosphate (pH 6.0) (total gradient volume 100 ml). Fractions were assayed for arabanase and arabinosidase activities as well as for protein content ($A_{280\ nm}$) (Fig. 1). Those fractions containing arabanase but no arabinosidase activity (fractions 75–112) were pooled and dialyzed against 10 mM Na-phosphate (pH 6.8).

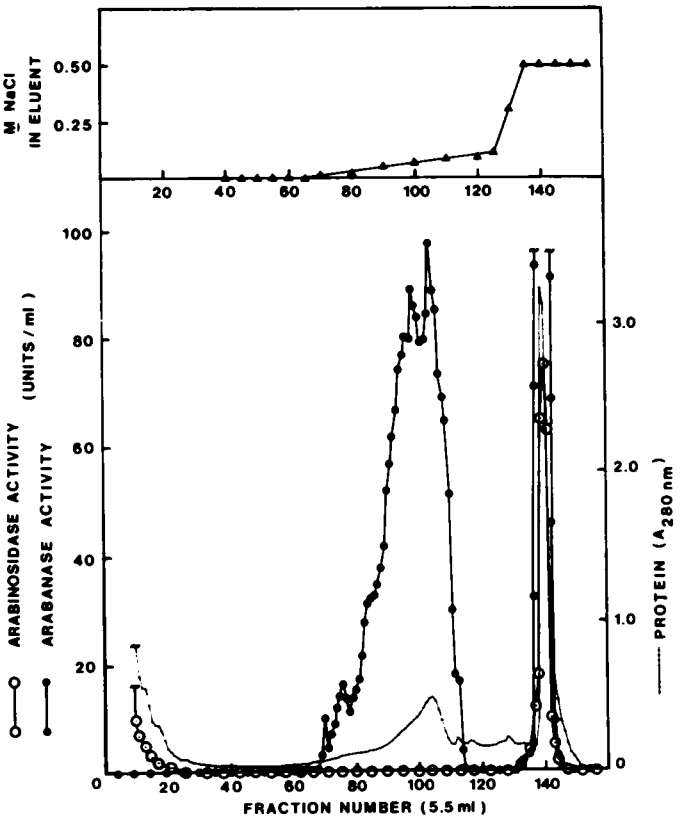


FIG. 1. CM-Sephadex C-50 chromatography (step 2) of the redissolved $(NH_4)_2SO_4$ precipitate. The column (2.5 × 27) was eluted with 10 mM Na-phosphate (pH 6.8). Linear gradients of increasing NaCl concentration were applied as described in the text. The volume of the fractions was 5.5 ml. The arabinosidase and the arabanase activity of each fraction was determined as described under "Experimental Procedure."

**Step 3. Hydroxylapatite Chromatography.** The pooled, dialyzed fraction obtained in step 2 was applied to a hydroxylapatite column (1.5 × 2.7 cm) previously equilibrated in 10 mM Na-phosphate (pH 6.8). The column was washed with 15 ml of 10 mM Na-phosphate (pH 6.8) followed by a linear phosphate buffer gradient from 10 to 100 mM (pH 6.8) (total gradient volume 200 ml) (Fig. 2). Fractions rich in arabanase activity (fractions 88–103) were pooled and stored at 0 C until further use.

**Step 4. Agarose 1.5m Chromatography.** A 1.0-ml aliquot of the preparation from the hydroxylapatite column (step 3) was applied to an agarose 1.5m column (1.5 × 70 cm), equilibrated in 10 mM Na-phosphate (pH 6.8). The column was eluted with this buffer, and the active fractions (Fig. 3) (fractions 37–43) were pooled and labeled "purified endo-arabanase." The endo-arabanase from the redissolved $(NH_4)_2SO_4$ precipitate (step 1) was purified about 204-fold, with 89% recovery of activity. The data summarizing the purification are in Table II.

### PURIFICATION OF ARABINOSIDASE II

Steps 1 and 2 used in the purification of arabinosidase II were identical to those used in the purification of the endo-arabanase. The fractions pooled from the CM-Sephadex C-50 chromatography step were those possessing the ability to catalyze the hydrolysis of both the branched araban and *p*-nitrophenyl-α-L-arabinofuranoside (Fig. 1, fractions 138–141). The pooled fractions obtained from CM-Sephadex chromatography were dialyzed against 10 mM Na-phosphate (pH 6.8) and loaded onto a hydroxylapatite column

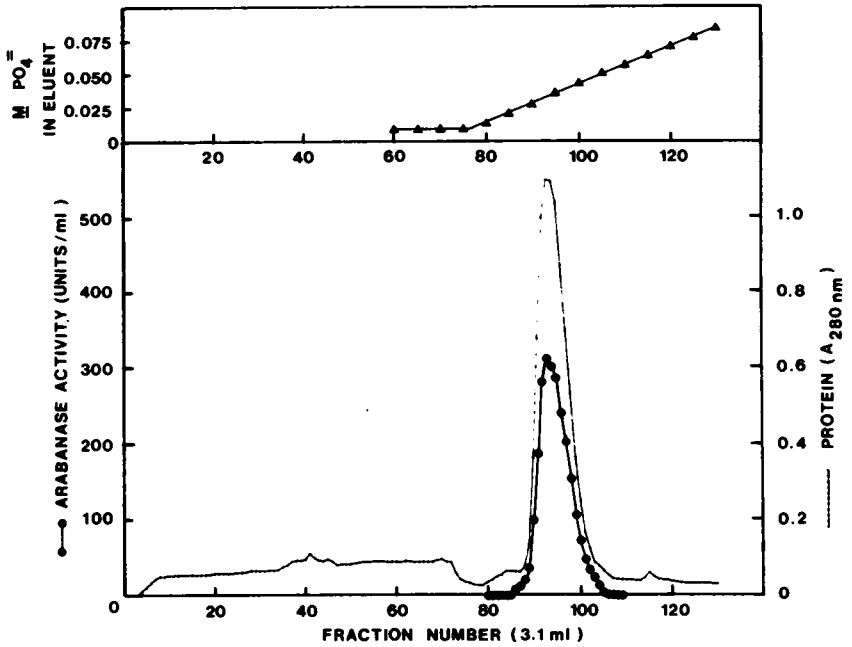


FIG. 2. Hydroxylapatite chromatography (step 3) of the CM-Sephadex C-50 purified endo-arabanase. The column (1.5 × 2.7 cm) was eluted with a linearly increasing Na-phosphate buffer gradient (10–100 mM) (pH 6.8). The volume of the fractions was 3.1 ml. The arabanase activity of each fraction was assayed as described under "Experimental Procedure."

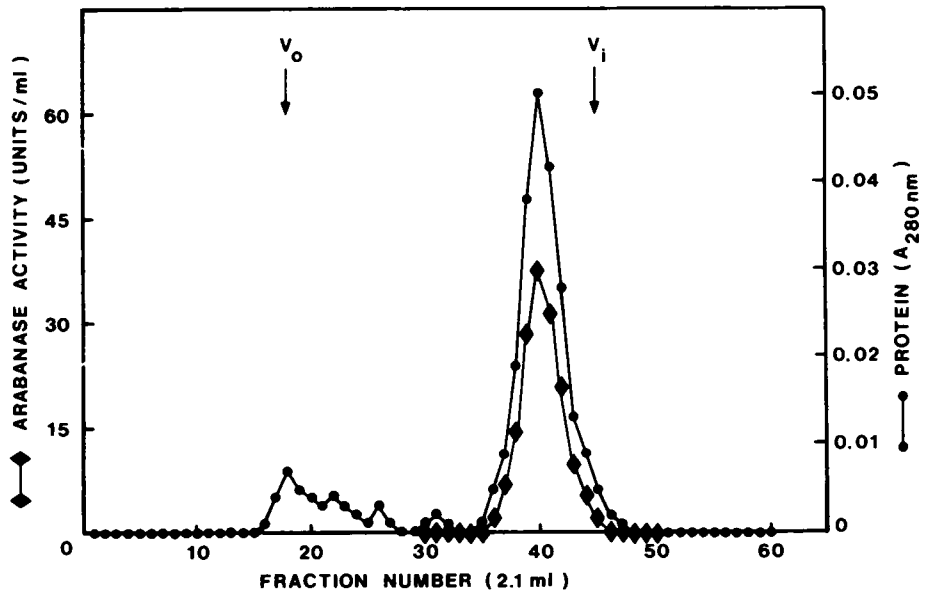


FIG. 3. Agarose 1.5m chromatography (step 4) of the hydroxylapatite purified endo-arabanase. The column (1.5 × 70 cm) was eluted with 10 mM Na-phosphate (pH 6.8). The volume of the fractions was 2.1 ml. Void and included volumes are indicated by $V_o$ and $V_i$, respectively. Arabanase activity was determined as described under "Experimental Procedure." Fractions 37 to 43 were pooled and termed "purified endo-arabanase."

(1.5 × 2.7 cm) previously equilibrated in 10 mM phosphate (pH 6.8). The hydroxylapatite column was eluted with a step gradient of Na-phosphate (pH 6.8). The gradient consisted of 10-ml steps starting at 100 mM phosphate and increasing in 100 mM increments to 1.0 M phosphate. The arabinosidase activity eluted in the 0.5 and 0.6 M phosphate washes. These fractions were pooled, dialyzed against 10 mM Na-phosphate (pH 6.0), and labeled purified arabinosidase II. The arabinosidase thus obtained was purified about 837-fold with about 35% recovery of activity. The data summarizing the purification are in Table III.

The use of CM-Sephadex chromatography affords a significant purification (Table II) of the endo-arabanase worthy of further comment. In actuality, three distinct arabinofuranosidic hydrolases are separated on this column. An enzyme possessing activity toward the *p*-nitrophenyl substrate but not toward the branched araban passes directly through the CM-Sephadex column (Fig. 1, fractions 0–20). This enzyme, termed arabinosidase I, has not been further studied. A second enzyme, capable of limited degradation of the branched araban but unable to hydrolyze *p*-nitrophenyl-α-L-arabinofuranoside, is eluted by a linear salt gradient (Fig. 1, fractions 75–112). This enzyme is apparently the endo-arabanase described by Kaji and Saheki (12). Arabinosidase II, capable both

Table II. Summary of purification of B. subtilis endo-arabanase.

| Fraction | Volume | Total arabanase activity | Yield | Total protein | Arabanase specific activity | Relative purification |
|---|---|---|---|---|---|---|
| | ml | units | % | mg | units/mg protein | |
| Redissolved $(NH_4)_2SO_4$ precipitate | 2850 | 8,800[a] | 100 | 4140[b] | 2.1 | 1 |
| CM-Sephadex C-50 | 21 | 8,500 | 97 | 31.5 | 270 | 129 |
| Hydroxylapatite chromatography | 49.6 | 8,000 | 91 | 21.3 | 376 | 179 |
| Agarose 1.5m | 729 | 7,800 | 89 | 18.2 | 429 | 204 |

[a] A calculated value; see text for discussion.

[b] Protein concentration in the redissolved $(NH_4)_2SO_4$ precipitate was determined by differential absorbance at 280 and 260 nm (17). Subsequent determinations were made by the procedure of Lowry et al. (18).

Table III. Summary of purification of B. subtilis arabinosidase II.

| Fraction | Volume | Total arabinosidase II activity | Yield | Total protein | Arabinosidase II specific activity | Relative purification |
|---|---|---|---|---|---|---|
| | ml | units | % | mg | units/mg protein | |
| Redissolved $(NH_4)_2SO_4$ precipitate | 2850 | 3,100[a] | 100 | 4,140[b] | 0.75 | 1 |
| CM-Sephadex C-50 | 22 | 1,600 | 52 | 37.5 | 42.7 | 57 |
| Hydroxylapatite chromatography | 31 | 1,070 | 35 | 1.7 | 628 | 837 |

[a] A calculated value; see text for discussion.

[b] Protein concentration in the redissolved $(NH_4)_2SO_4$ precipitate was determined by differential absorbance at 280 and 260 nm (17). Subsequent determinations were made by the procedure of Lowry et al. (18).

of hydrolyzing the *p*-nitrophenyl-α-L-arabinofuranoside and of partial hydrolysis of the branched araban, is eluted by still higher salt concentrations (Fig. 1, fractions 138–141).

### ESTIMATE OF AMOUNT OF ENDO-ARABANASE AND ARABINOSIDASE II PRESENT IN REDISSOLVED $(NH_4)_2SO_4$ PRECIPITATE

The redissolved $(NH_4)_2SO_4$ precipitate contained all three arabinoside hydrolases. Since two of these enzymes, the endo-arabanase and arabinosidase II, can hydrolyze the branched araban, the value for amount of endo-arabanase activity present in the redissolved $(NH_4)_2SO_4$ precipitate had to be estimated. Addition of purified arabinosidase II to the purified endo-arabinase results in an increase in the "apparent" arabanase activity on the branched araban substrate. The rate of hydrolysis of the branched araban is stimulated a maximum of 4-fold when the ratio of arabinosidase II units to arabanase units is greater than 0.1. In fact, the redissolved $(NH_4)_2SO_4$ precipitate has an arabinosidase II to arabanase activity ratio of about 0.35 (Tables II and III), far greater than required for maximal (4-fold) stimulation of branched araban degradation. The amount of arabanase activity present in the redissolved $(NH_4)_2SO_4$ precipitate is estimated to be one-quarter of the originally observed value of 35,200 units, *i.e.* about 8,800 units. The approximated value of 8,800 arabanase units present in the redissolved $(NH_4)_2SO_4$ precipitate indicates that 89% of the arabanase activity present in the redissolved $(NH_4)_2SO_4$ precipitate is recovered in the purified enzyme.

The amount of arabinosidase II present in the redissolved $(NH_4)_2SO_4$ precipitate also has to be approximated because of the presence of arabinosidase I which, like arabinosidase II, is capable of hydrolyzing *p*-nitrophenyl-α-L-arabinofuranoside. Arabinosidase II, but not arabinosidase I, is capable of hydrolyzing the branched araban. An estimation of the amount of arabinosidase II present in the redissolved $(NH_4)_2SO_4$ precipitate fraction can be obtained from the observed rate of hydrolysis of the araban. One unit of arabinosidase II (free of arabinosidase I), as assayed on the *p*-nitrophenyl-α-L-arabinofuranoside, is equivalent to 8.6 units of this enzyme when assayed on the branched araban. Subtracting the previously estimated contribution of the endo-arabanase (8,800 units) from the observed 35,200 units of "arabanase" activity in the redissolved $(NH_4)_2SO_4$ precipitate, the amount of arabinosidase II present in this fraction is estimated to be 26,400 units when assayed on the araban as substrate, or about 3,100 units when assayed using *p*-nitrophenyl-α-L-arabinofuranoside as substrate. If this amount of arabinosidase II is present in the redissolved $(NH_4)_2SO_4$ precipitate, then 35% of the purified arabinosidase II is recovered.

### CHARACTERIZATION OF PURIFIED ENDO-ARABANASE AND ARABINOSIDASE II

**Mol Wt Determinations.** The mol wt of the purified endo-arabanase was determined by gel filtration chromatography as well as by SDS-gel electrophoresis in 7 M urea. Chromatography of the endo-arabanase on a calibrated agarose 1.5m column (1.5 × 70 cm) in 10 mM Na-phosphate (pH 6.8) indicated a mol wt of 36,000, whereas SDS-urea gel electrophoresis of 30 μg of purified endo-arabinase showed a single protein band corresponding to a mol wt of 32,000 by comparison to standards (see under "Experimental Procedure").

The mol wt of purified arabinosidase II was estimated by SDS disc gel electrophoresis in 7 M urea. Upon electrophoresis and subsequent staining of the gel, one dark protein band was observed at a mol wt of about 65,000, accompanied by a much fainter band at a mol wt of about 58,000. Gel exclusion chromatography was attempted in agarose 1.5m; however, the enzyme was found to aggregate under a variety of experimental conditions. Further attempts to size arabinosidase II on the agarose column were not attempted.

**Isoelectric Focusing of Purified Enzymes.** Isoelectric focusing

of the purified endo-arabanase gives rise to a single protein band at pH 9.3. Using the zymogram technique, as described by Eriksson and Pettersson (6), a single band of arabanase activity is observed which coincides with the protein band at pH 9.3.

Isoelectric focusing of purified arabinosidase II gives rise to two bands, a dark diffuse band at pH 5.3, and a faint, but sharp band at pH 8.1. Activity toward the araban substrate was again located using the zymogram technique (6); the heavily stained protein band at pH 5.3 possesses the glycosidase activity.

**pH Optima of Purified Enzymes.** The effect of pH upon the ability of the purified endo-arabanase to hydrolyze the branched araban is shown in Figure 4A. The endo-arabanase exhibits maximum activity at about pH 6.0, in agreement with the pH optimum observed by Kaji and Saheki (12). The pH optimum of arabinosidase II on the *p*-nitrophenyl-α-L-arabinofuranoside substrate is shown (Fig. 4B) to be at about pH 6.5.

**Evaluation of Divalent Cation Requirements.** Purified endo-arabanase and arabinosidase II were dialyzed for 72 hr against 10 liters 5 mM EDTA in 10 mM Na-phosphate (pH 6.8) followed by dialysis for 72 hr against 10 liters 10 mM Na-phosphate (pH 6.8) to remove the EDTA. The activities of both the endo-arabanase and arabinosidase II did not change appreciably due to the dialysis against EDTA, suggesting that neither enzyme possesses a divalent cation requirement.

**Examination for Presence of Other Glycoside Hydrolases in Purified Enzyme Preparations.** A major criterion that must be satisfied by any enzyme preparation to be used in determining the structure of primary cell wall polysaccharides is that the preparation does not hydrolyze glycosidic linkages in any wall polysaccharides other than the intended substrate. We have assayed the purified endo-arabanase and arabinosidase II for their ability to hydrolyze the other glycosidic linkages commonly present in primary cell wall polysaccharides. The model substrates tested included polygalacturonic acid, containing the α-1,4-D-galacturonopyranosidic linkages found in cell wall rhamnogalacturonans (24); carboxymethylcellulose, containing the β-1,4-glucopyranosidic linkages found in cellulose and in xyloglucan (3); larch galactan, containing the β-D-galactopyranosidic linkages found in 3,6-linked galactan (14); citrus pectic galactan, containing the β-1,4-D-galactopyranosidic linkages of another cell wall galactan (24); and complex beet araban, containing the variety of arabinosyl linkages characteristic of wall araban (24). Other potential substrates included the *p*-nitrophenyl derivatives of α- and β-D-xylopyranosides, α- and β-D-glucopyranosides, β-D-fucopyranoside, and β-D-galactopyranoside. In these experiments, 0.55 units of purified endo-arabanase or 0.43 units of purified arabinosidase II were incubated for 6 hr with one of the potential substrates (2.0 mg/ml in 10 mM Na-phosphate, pH 6.0). The assays were terminated by immediate determination of either reducing groups or *p*-nitrophenol. No contaminating enzymic activity could be detected in either of the two purified enzyme preparations.

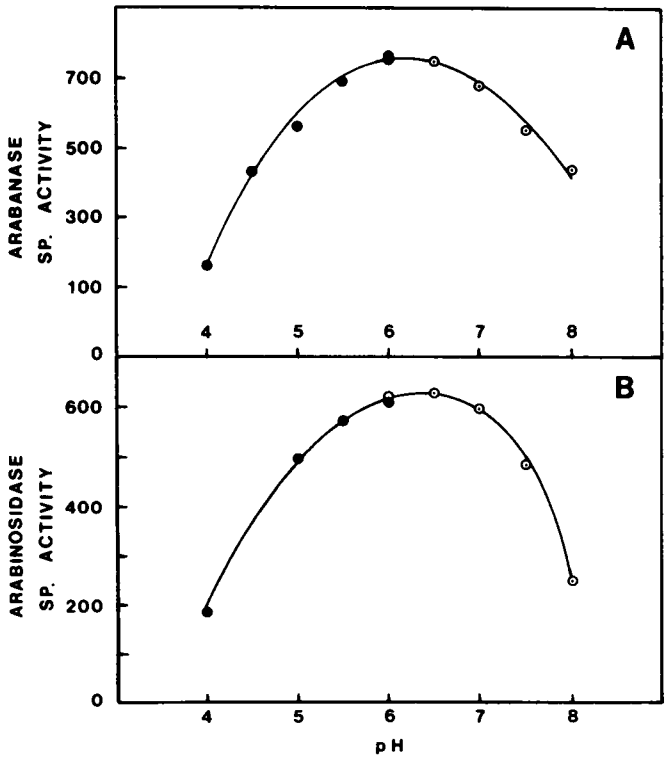


FIG. 4. Specific activity of endo-arabanase (A) and arabinosidase II (B) as a function of pH. Reaction mixtures contained either 2 mg/ml of purified araban or 2 mg/ml of *p*-nitrophenyl-α-L-arabinofuranoside in 10 mM acetate (●) or 10 mM Na-phosphate (○). Assays were carried out as described under "Experimental Procedure." The pH measurements were made with a Beckman research pH meter just prior to termination of the assay.

**Substrate Specificity and Mode of Attack of Purified Endo-Arabanase and Arabinosidase II.** The branched araban is actually a poor substrate for the endo-arabanase. This enzyme is capable of hydrolyzing a maximum of about 3% of the glycosidic linkages (100% hydrolysis was based upon the reducing group ($A_{520\ nm}$) (20, 22) value observed upon hydrolysis of the branched araban with 2 N trifluoroacetic acid at 121 C for 1 hr).

The purified branched araban still contains about 3% galactose. Therefore, the possibility existed that a galactanase, and not an endo-arabanase, had been isolated. In order to verify that an endo-arabanase had indeed been isolated, the following experiment was performed. Purified araban (50 mg) in 2 ml of 10 mM Na-phosphate (pH 6.0) was incubated for 48 hr with 0.55 units of purified endo-arabanase. Toluene (5 μl) was added to retard bacterial growth. The reaction was terminated by placing the reaction mixture in a boiling water bath for 15 min to denature the endo-arabanase. The heated hydrolysis mixture was applied directly to a Bio-Gel P-2 column (1.4 × 113 cm) which had been equilibrated with and which was eluted with deionized $H_2O$ at 50 C (Fig. 5). The orcinol-positive material of fractions 87 to 91 was pooled and lyophilized.

The reducing ends of the oligomers present in fractions 87 to 91 (Fig. 5) were reduced with Na-borohydride, and the reduced oligosaccharides analyzed for their neutral sugar composition by formation of the monodeuterium-labeled alditol acetates (2). The arabinosyl residues not hydrolyzed by the enzyme were subsequently reduced with Na-borodeuteride rather than Na-borohydride in order to discriminate unhydrolyzed residues from those that had been hydrolyzed by the endo-arabanase. Gas chromatography of the alditol acetates derived from the oligosaccharides present in fractions 87 to 91 demonstrated that arabinose is the only glycosyl residue present in the isolated oligomer(s).

Chemical ionization (CI) MS of the alditol acetates derived from fractions 87 to 91 gave rise to two ions, one at m/e 303 and another, twice as large, at m/e 304. The m/e 303 ion represents the 1,2,3,4,5-O-penta-acetyl arabinitol minus 60 (acetic acid) which originates from the reducing end of the oligosaccharide, the end reduced with Na-borohydride. The m/e 304 ion represents the 1-deutero-1,2,3,4,5-O-penta-acetyl arabinitol minus 60 (acetic acid) which originates from the arabinosyl residues not at the reducing ends of the oligosaccharide and therefore reduced with Na-borodeuteride. The 2:1 ratio of the 304/303 ions indicates that a trimer of arabinose was present in fractions 87 to 91. These data prove the enzyme in question to be an arabanase. Methylation analysis of all of the orcinol-positive peaks released by the action of the endo-arabanase on the araban and resolved upon P-2 column chromatography (Fig. 5), including the residual polymeric araban left at the void fractions, gave little additional information concerning the mode of attack by the endo-arabanase. Only about 3% of the available linkages in the substrate were hydrolyzed by

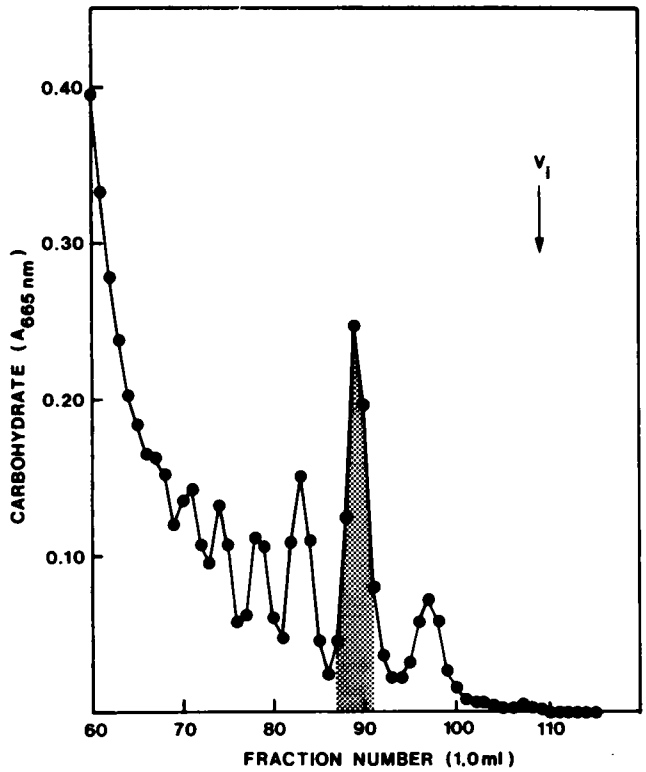


FIG. 5. Bio-Gel P-2 fractionation of the products of endo-arabanase attack on purified araban. A sample of purified araban was treated with endo-arabanase and then chromatographed on a Bio-Gel P-2 (200–400 mesh) column (1.4 × 113 cm) operated at 50 C and eluted with water. Fractions of 1.0 ml were collected at a flow rate of 0.2 ml/min. A 50-μl aliquot of each fraction was assayed for carbohydrate by the orcinol test (4). Purified araban, prior to treatment with endo-arabanase, completely voids the column. The peak at fractions 87 to 91, indicated by shading, was subjected to the analysis described in the text. This peak contains a trimer of arabinose.

these exhaustive enzymolysis conditions. It was difficult to determine if any of the arabinosyl linkages present in the branched substrate had been preferentially attacked. However, linkage analysis of the oligomers generated by the endo-arabanase demonstrated that all of the reducing ends produced and detected were 5-linked arabinose.

Additional information regarding the mode of attack of the endo-arabanase was obtained by analysis of the action of endo-arabanase on the debranched araban substrate. The purified endo-arabanase was capable of hydrolyzing the partially debranched araban at about 16 times the initial rate of hydrolysis of the highly branched araban. Previous reports (9–11) indicate that beet araban contains a linear 5-linked araban backbone with 2- and 3-linked arabinosyl side chains. Therefore, the increased rate of hydrolysis of the debranched araban over the araban was taken as evidence that the endo-arabanase attacks the 5-linked backbone of the araban substrate, and that the presence of branched chains of 2- and 3-linked arabinosyl residues interfere with the enzyme's accessibility to its substrate.

Evidence will now be presented that the enzyme termed endo-arabinase does indeed attack its substrate in an endo- rather than an exo- fashion. Experiments were performed to observe the rate of appearance of the arabanase-produced short oligomers of arabinose. Debranched araban (20 mg) was incubated at 25 C with 0.22 units of purified endo-arabanase in 2.0 ml 10 mM Na-phosphate (pH 6.0). Toluene (5 μl) was added to retard bacterial growth. Aliquots of 0.5 ml were removed at 30-min intervals, placed in a boiling water bath for 15 min to denature the endo-arabanase, and applied to a Bio-Gel P-2 column (1.4 × 113 cm) which had been equilibrated with and which was eluted with deionized $H_2O$ at 50 C. Chromatography of the various aliquots on the P-2 column (Fig. 6) indicated a gradual appearance of different size oligomers that decrease in average size with increasing exposure to the endo-arabanase. The pattern observed is consistent with the endo-arabanase attacking the araban in an endo- fashion, and supports the conclusion of Kaji and Saheki (12) that the arabanase is an endo-glycanase.

Purified arabinosidase II was suspected as being an exo-glycanase since it does hydrolyze the *p*-nitrophenyl-α-L-arabinoside substrate. This was further substantiated by analysis of the arabinosidase II degradation products of the branched araban.

Purified araban (20 mg) was incubated at 25 C in 2.0 ml 10 mM Na-phosphate (pH 6.5) with 0.34 units purified arabinosidase II. Aliquots (0.5 ml) were removed at 15-min intervals and subjected to the same product analysis as for the endo-arabanase. Regardless of the extent of hydrolysis, the only product observed to be released by arabinosidase II was monomeric arabinose, consistent with the notion that arabinosidase II is an exo- degrading enzyme.

**Action of Purified Endo-Arabanase and Arabinosidase II on Suspension-cultured Sycamore Cell Walls.** Cell walls were prepared from suspension-cultured sycamore cells and freed of starch by α-amylase, as previously described (24). Samples (2.0 mg) of lyophilized, starch-free cell walls were suspended in 0.95 ml of 10 mM Na-phosphate (pH 6.0) containing 0.03 units of endo-arabanase and/or arabinosidase II. The reaction mixture was allowed to incubate for 3 hr in the presence of 5 μl of toluene acting as a bacteriostat. Following incubation, residual solid material was removed by centrifugation, and 0.5 ml of the resulting supernatant

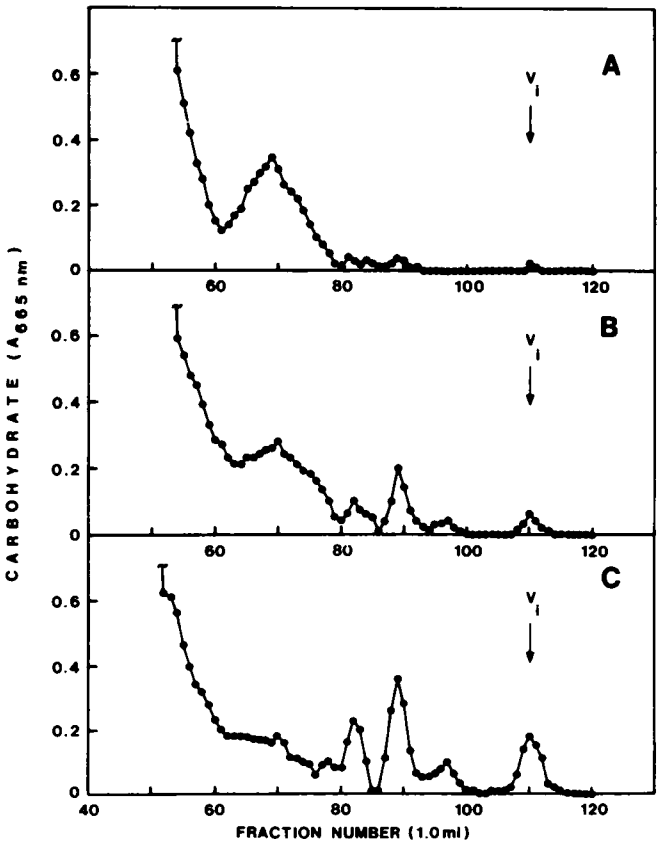


FIG. 6. Bio-Gel P-2 fractionation of the products of endo-arabanase attack on debranched araban. The reactions were carried out as described in the text. Details of the separations can be found in the legend of Figure 5. A: 15-min hydrolysis; B: 30-min hydrolysis; C: 60-min hydrolysis. Debranched araban, prior to treatment with endo-arabanase, completely voids the column.

solution was removed for neutral sugar analysis (2). Only arabinose oligomers are solubilized by the endo-arabanase. Arabinosidase II, by itself, has little or no effect on the cell wall suspension. The endo-arabanase, used either alone or in conjunction with arabinosidase II, is capable of releasing 40 μg of araban from the cell walls which contain approximately 200 μg of arabinose before exposure to the enzyme. The fact that arabinosidase II apparently has little, if any, activity against primary cell walls could be due to inaccessibility of wall araban. However, purified arabinosidase II will certainly find applicability as a secondary treatment of solubilized wall arabans to assist in the further elucidation of their structure.

## CONCLUSIONS

Two of three detected arabinoside hydrolases secreted by *B. subtilis* F-11 into its culture medium have been purified and shown to be an endo-arabanase and an arabinosidase. These enzymes possess the ability to partially degrade beet araban and the arabinosyl linkages present in primary cell wall polysaccharides. The endo-arabanase and arabinosidase have been purified to the point where they contain no detectable contaminating enzymic activities. These enzymes will be useful in determining the structure of the polymeric araban components of primary cell walls.

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