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An *Aspergillus niger* Esterase (Ferulic Acid Esterase III) and a Recombinant *Pseudomonas fluorescens* subsp. *cellulosa* Esterase (XylD) Release a 5-5′ Ferulic Dehydrodimer (Diferulic Acid) from Barley and Wheat Cell Walls

BEGOÑA BARTOLOMÉ,[1]† CRAIG B. FAULDS,[1] PAUL A. KROON,[1] KEITH WALDRON,[2] HARRY J. GILBERT,[3] GEOFF HAZLEWOOD,[4] AND GARY WILLIAMSON[1]*

Department of Biochemistry[1] and Department of Food Biophysics,[2] Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA, and Department of Biological and Nutritional Sciences, University of Newcastle, Newcastle upon Tyne, NE1 7RU,[3] and Department of Cellular Physiology, Babraham Institute, Babraham, Cambridge CB2 4AT,[4] United Kingdom

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Diferulate esters strengthen and cross-link primary plant cell walls and help to defend the plant from invading microbes. Phenolics also limit the degradation of plant cell walls by saprophytic microbes and by anaerobic microorganisms in the rumen. We show that incubation of wheat and barley cell walls with ferulic acid esterase from *Aspergillus niger* (FAE-III) or *Pseudomonas fluorescens* (XylD), together with either xylanase I from *Aspergillus niger*, *Trichoderma viride* xylanase, or xylanase from *Pseudomonas fluorescens* (XylA), leads to release of the ferulate dimer 5-5′diFA [(E,E)-4,4′-dihydroxy-5,5′-dimethoxy-3,3′-bicinnamic acid]. Direct saponification of the cell walls without enzyme treatment released the following five identifiable ferulate dimers (in order of abundance): (Z)-β-{4-[(E)-2-carboxyvinyl]-2-methoxyphenoxy}-4-hydroxy-3-methoxycinnamic acid, *trans*-5-[(E)-2-carboxyvinyl]-2-(4-hydroxy-3-methoxy-phenyl)-7-methoxy-2,3-dihydrobenzofuran-3-carboxylic acid, 5-5′diFA, (E,E)-4,4′-dihydroxy-3,5′-dimethoxy-β,3′-bicinnamic acid, and *trans*-7-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-6-methoxy-1,2-dihydronaphthalene-2,3-dicarboxylic acid. Incubation of the wheat or barley cell walls with xylanase, followed by saponification of the solubilized fraction, yielded 5-5′diFA and, in some cases, certain of the above dimers, depending on the xylanase used. These experiments demonstrate that FAE-III and XYLD specifically release only esters of 5-5′diFA from either xylanase-treated or insoluble fractions of cell walls, even though other esterified dimers were solubilized by preincubation with xylanase. It is also concluded that the esterified dimer content of the xylanase-solubilized fraction depends on the source of the xylanase.

Primary cell walls of members of the family *Graminaceae* contain FA and other hydroxycinnamic acids ester linked to arabinosyl residues in arabinoxylans (20). A proportion of these phenolics are present as dimers which function to cross-link and strengthen the cell wall (4, 12). Several dimeric structures have been identified, and the linkages can be through either the phenolic ring or the unsaturated aliphatic side chain (18) (Fig. 1). FAEs release FA from arabinoxylans, which increases the susceptibility of the cell wall to further enzymatic attack (1). Only certain FAEs can act alone on a preparation of the cell wall, such as wheat bran, to release FA (6). Others require the additional activity of a xylanase, and the esterase activity has so far been increased by xylanases (3, 7). Xylanases solubilize feruloylated compounds (such as FAXX and other feruloylated oligosaccharides), which are better substrates for FAE than the insoluble cell wall (7). There are, however, no reports identifying enzymes that are able to hydrolyze phenolic dimers from arabinoxylans, either in the presence or in the absence of other enzymes, such as xylanases. Dimers can be released chemically by saponification, and this is the method by which cell wall FA dimers have been identified and characterized. Since the dimers play an important structural role in some plant cell walls (12), enzymatic hydrolysis of dimers would be expected to alter the physical properties of the wall and make it more accessible to further enzymatic attack. In this report, we show that two esterases, FAE-III from *Aspergillus niger* (6) and XylD from *Pseudomonas fluorescens* (8), alone or more effectively in concert with a xylanase, release a diFA (5-5′diFA) from barley and wheat cell walls. Although other dimers are present, they are not released by these esterases.

* Corresponding author. Department of Biochemistry, Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA, U.K. Phone: 44 1603 255259. Fax: 44 1603 507723. E-mail: GARY.WILLIAMSON@BBSRC.AC.UK.

† Present address: Instituto de Fermentaciones Industriales (CSIC), Juan de la Cierva 3, Madrid, 28006 Spain.

MATERIALS AND METHODS

Abbreviations. 8-8′ArylD, *trans*-7-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-6-methoxy-1,2-dihydronaphthalene-2,3-dicarboxylic acid; 5-8′BenDi, *trans*-5-[(E)-2-carboxyvinyl]-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-2,3-dihydrobenzofuran-3-carboxylic acid; 8-*O*-4′diFA, (Z)-β-{4-[(E)-2-carboxyvinyl]-2-methoxyphenoxy}-4-hydroxy-3-methoxycinnamic acid; 5-5′diFA, (E,E)-4,4′-dihydroxy-5,5′-dimethoxy-3,3′-bicinnamic acid; 5-8′diFA, (E,E)-4,4′-dihydroxy-3,5′-dimethoxy-β,3′-bicinnamic acid; FA, ferulic acid; FAE, FA esterase; FAXX, *O*-[5-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl]-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose; HPLC, high-performance liquid chromatography; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; xylanase, β-(1,4)-D-xylan xylanohydrolase (EC 3.2.1.8.); XylA, xylanase from *P. fluorescens* subsp. *cellulosa*; XylD, esterase from *P. fluorescens* subsp. *cellulosa*; FAE-III, *A. niger* FAE; xylanase I, xylanase from *A. niger*.

Enzymes. FAE-III was purified as described previously (6). Recombinant XylD and XylA, expressed in *Escherichia coli*, were obtained and purified as described previously (8, 10). *Trichoderma viride* xylanase (99% pure) was purchased from Megazyme Pty. Ltd. Xylanase I (11) was a gift from M. Tuohy (Department of Biochemistry, University College, Galway, Ireland).

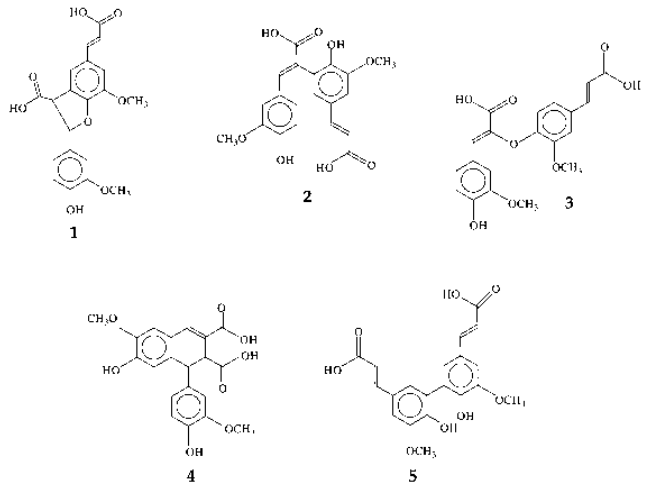

FIG. 1. Structures of some FA dehydrodimers. 1, 5-8′BenDi; 2, 5-8′diFA; 3, 8-*O*-4′diFA; 4, 8-8′ArylD; 5, 5-5′diFA.

Substrates. Spent grain from *Puffin* variety barley was a gift from C. Bamforth (Brewing Research Foundation International, Surrey, United Kingdom). This material was freeze-dried and milled to a fine powder with a particle size of less than 50 μm. This sample contained 0.32% (wt/wt) esterified (mono)FA. Spent grain contained 21% protein, 11% lipids, 11% cellulose, 6.5% lignin, 44% glucans plus pentosans, and 1.4% free sugars, expressed on a dry-weight basis. Destarched wheat bran was made as previously described (1) and milled to the same particle size as the spent grain. Wheat bran contained 16% protein, 19% starch, 2.6% lignin, 26% arabinoxylan, and other carbohydrates which constituted 6.6% uronic acids, 1.3% mannose, 1.2% galactose, and 11% glucose after hydrolysis.

Enzyme assays. FAE activity was determined by measuring the rate of hydrolysis of methyl FA (5). One unit of activity is defined as the amount of enzyme releasing 1 μmol of FA min^{-1} at pH 6.0 and 37°C. Xylanase activity was estimated by measuring the release of reducing sugars (16) from soluble oat spelt xylan (1%, wt/vol) (15). One unit of activity is defined as the amount of enzyme releasing 1 μmol of sugar min^{-1} at pH 5.3 and 37°C.

Chemical hydrolysis. Alkali hydrolysis of both spent grain and wheat bran was carried out by adding NaOH (0.55 ml of 1.0 M) to samples (10 mg), followed by incubation at 20°C for 16 h under N_2. After centrifugation (13,000 × *g*, 15 min), the supernatant was collected, acidified with HCl to pH 3 to 3.5, and extracted five times with equal volumes of ethyl acetate. The organic fractions were combined and evaporated to dryness, and the residue was dissolved in the mobile phase (0.5 ml) (see HPLC conditions). Samples were prepared and analyzed in duplicate.

Enzymatic hydrolyses. Enzymatic hydrolyses were carried out in MOPS buffer (0.1 M, pH 6.0) in a thermostatically controlled rotating incubator, at 37°C, for 7 h. Samples (10 mg) were incubated with esterase (1.3 U/g) in the presence and in the absence of xylanase (200 U/g) in a final volume of 0.55 ml. Some samples were pretreated with xylanase and then centrifuged (13,000 × *g*, 15 min). The resulting supernatant was then treated with either NaOH solution (final concentration, 1.0 M) at 20°C for 16 h under N_2 or with esterase at 37°C for 7 h. After incubation, enzymatic reactions were stopped by boiling for 3 min. The samples were cooled and centrifuged (13,000 × *g*, 5 min). Determination of the content of FA and FA dehydrodimers in the supernatants, after extraction with ethyl acetate as described above, was done by HPLC. Samples were prepared and analyzed in duplicate.

HPLC conditions. HPLC was performed as described previously (22). Extracted phenolic acids were dissolved in 50% (vol/vol) methanol; *trans*-cinnamic or *m*-coumaric acid was added as an internal standard. Samples were loaded onto an Interpak ODS2 5-μm reverse-phase column (250 by 5 mm; Capital HPLC Ltd., Broxburn, West Lothian, United Kingdom), with gradient elution employing progressively increasing methanol-acetonitrile levels in 1 mM trifluoroacetic acid. Phenolics were detected and quantified with a Perkin Elmer LC-235C diode array detector, and the identity of each peak was confirmed by spectral analysis (22). The limits of detection all of the dimers, their response factors, and their spectra have been reported by Waldron et al. (22).

RESULTS

Barley and wheat contain relatively high levels of FA (1, 2). Two cell wall materials derived from spent barley grain and destarched wheat bran were analyzed for total alkali-extractable feruloylated compounds. FA and the following dimers were found in the supernatants of spent grain and wheat bran after NaOH hydrolysis (Fig. 2 and 3): 8-8′ArylD, 5-8′diFA, 5-5′diFA, 8-*O*-4′diFA, and 5-8′BenDi. In both spent grain and wheat bran, the order of abundance was 8-*O*-4′diFA > 5-8′BenDi > 5-5′diFA > 5-8′diFA > 8-8′ArylD (Tables 1 and 2). Although wheat bran showed a higher absolute dimer content than spent grain, relative FA dimerization (the ratio of total dimers to monomers) was about twofold higher in barley spent grain.

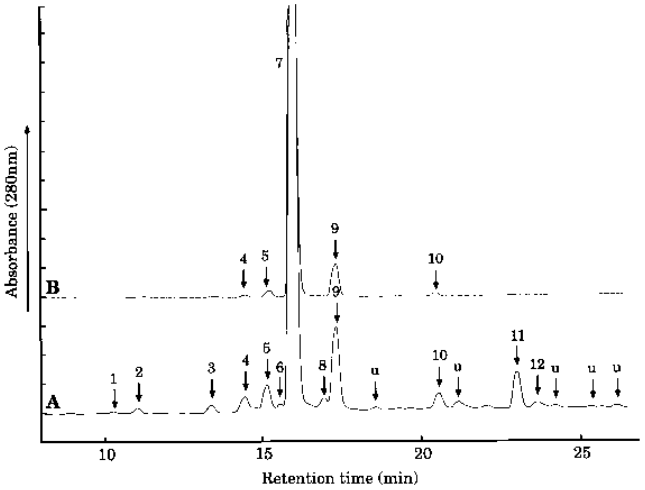

FIG. 2. HPLC chromatograms (at 310 nm) of the supernatants of spent barley grain (0.01 g) after NaOH hydrolysis at 20°C for 16 h under N_2 (A) and after incubation with *A. niger* FAE-III (1.3 U/g) and *T. viride* xylanase (200 U/g) at 37°C, in MOPS, pH 6.0 (B). 1, *p*-hydroxybenzoic acid; 2, vanillic acid; 3, *p*-hydroxybenzaldehyde; 4, vanillin; 5, (*trans*)-*p*-coumaric acid; 6, 8-8′ArylD; 7, *trans*-FA; 8, 5-8′diFA; 9, *cis*-FA; 10, 5-5′diFA; 11, 8-*O*-4′diFA; 12, 5-8′BenDi; i, internal standard; u, unidentified compound with a spectrum typical of FA dehydrodimers. For clarity of presentation, the internal standard is shown in only one of the chromatograms (see Fig. 3).

The ability of FAE-III and XylD to release dehydrodimers from cell wall materials was investigated. We were not able to detect the release of FA dehydrodimers in incubations of spent

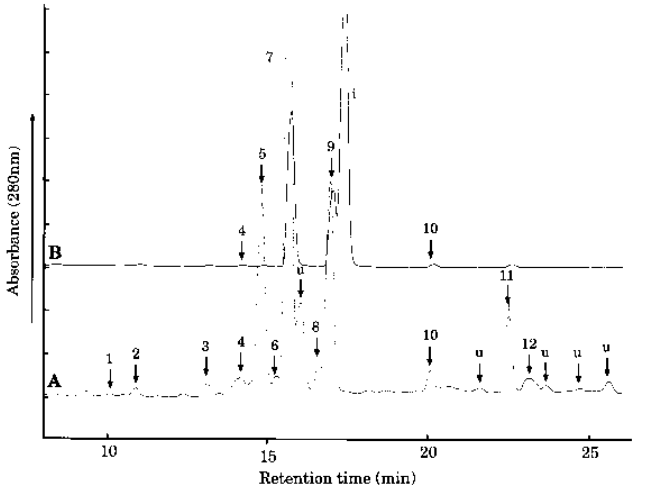

FIG. 3. HPLC chromatograms (at 310 nm) of the supernatants of destarched wheat bran (0.01 g) after NaOH hydrolysis at 20°C for 16 h under N_2 (A) and after incubation with XylD (1.3 U/g) and *T. viride* xylanase (200 U/g) at 37°C in MOPS, pH 6.0 (B). Peak labels are the same as in FIG. 2.

TABLE 1. Free FA and FA dehydrodimers released from spent barley grain by alkali and enzymatic treatments

First treatment (concn)	Subsequent treatment of solubilized fraction (concn)	Release[a] of:					
		FA	8-8′ ArylD	5-8′ diFA	5-5′ diFA	8-*O*-4′ diFA	5-8′ BenDi
NaOH (1.0 M)	None	15.5	TR	0.4	0.42	1.84	0.86
FAE-III (1.3 U/g)	None	0.66					
XylD (1.3 U/g)	None	1.43			0.025		
FAE-III (1.3 U/g) + *T. viride* xylanase (200 U/g)	None	4.86			0.075		
XylD (1.3 U/g) + *T. viride* xylanase (200 U/g)	None	4.21			0.060		
T. viride xylanase (200 U/g)	FAE-III (1.3 U/g)	5.29			0.10		
T. viride xylanase (200 U/g)	XylD (1.3 U/g)	4.55			0.10		
T. viride xylanase (200 U/g)	NaOH (1.0 M)	6.66	TR	0.05	0.094	0.31	0.054

[a] Data are means of duplicate analyses and are expressed as μmoles of compound released per gram of starting material. TR, trace.

grain or wheat bran with FAE-III (1.3 U/g), although some 5,5′diFA was released from wheat bran in the presence of FAE-III at 13 U/g (Tables 1 and 2). XylD (1.3 U/g) released a small amount of 5-5′diFA from spent grain but not from wheat bran. Both enzymes also released free FA from spent grain and wheat bran. XylD showed slightly higher FAE activity than FAE-III on the substrates under these conditions.

When cell wall materials were incubated with either of the esterases (1.3 U/g) in the presence of *T. viride* xylanase (200 U/g), a large peak identified as 5-5′diFA was observed (Fig. 2 and 3). Very small differences were seen between the amounts of 5-5′diFA released by FAE-III and XylD from spent grain (Table 1) or from wheat bran (Table 2). The total amount released was about 16% of the total alkali-extractable 5-5′diFA from spent grain and about 20% of the total alkali-extractable 5-5′diFA from wheat bran. As reported in previous studies (2, 7), the amount of free FA released by the esterases increased dramatically (up to ~70-fold) in the presence of the xylanase. These results suggest that the action of the xylanase solubilizes part of the cell wall structure. The resulting soluble feruloylated oligosaccharides and diferuloylated (di)oligosaccharides were then hydrolyzed by the esterase to produce free FA and FA dehydrodimers. To confirm this, spent grain and wheat bran were pretreated with *T. viride* xylanase (200 U/g), the resulting mixture was centrifuged, and the solubilized material was incubated with esterase (1.3 U/g). No free (i.e., not esterified) FA and no free FA dehydrodimers were solubilized by xylanase pretreatment. However, after xylanase pretreatment followed by esterase treatment, FA and 5-5′diFA were released. The amount of 5,5′diFA released was very similar to that obtained by the simultaneous incubations with esterase and xylanase described above (Tables 2 and 3). This implies that there is no biproduct heterosynergy (also called reciprocal cooperation) between the esterase and xylanase for the release of 5,5′diFA, although this effect is seen for the release of (mono)FA (1).

The rate of hydrolysis by xylanase and FAE-III of diferuloylated (di)oligosaccharides relative to (mono)feruloylated oligosaccharides and the extent of enzymic release of 5-5′diFA were measured (Fig. 4). Both *trans*-FA and 5-5′diFA were rapidly released from the solubilized feruloylated material by FAE-III. The release of FA was most rapid over the 0- to 10-min period and slower over the 10- to 60-min period. After this time, release of FA reached a maximum level of 450 nmol/g of sample, which accounted for 64% of the xylanase-solubilized, alkali-extractable FA in the sample. Release of 5-5′diFA had a profile similar to that of *trans*-FA, except that the amount of free acid released still increased slowly after 120 min. The 1.53 nmol of 5-5′diFA released after 120 min accounted for 77% of the xylanase-solubilized, alkali-extractable 5-5′diFA present in the sample.

The esterase and xylanase together, therefore, release only free (mono)FA and the 5,5′-diFA dimer. Since other dimers were released by alkali treatment of the starting materials, we carried out further experiments to determine which enzyme limited the release of other dimers. *T. viride* xylanase was used to pretreat destarched wheat bran and spent barley grain as described above, and the solubilized material was prepared by centrifugation. Instead of esterase hydrolysis of the solubilized fraction, alkali hydrolysis was performed. The alkali-hydro-

TABLE 2. Free FA and FA dehydrodimers released from destarched wheat bran by alkali and enzymatic treatments

First treatment (concn)	Subsequent treatment of solubilized fraction (concn)	Release[a] of:					
		FA	8-8′ ArylD	5-8′ diFA	5-5′ diFA	8-*O*-4′ diFA	5-8′ BenDi
NaOH (1.0 M)	None	40.8	TR	0.66	0.74	2.28	0.96
FAE-III (1.3 U/g)	None	0.45					
FAE-III (13 U/g)	None	10.8			0.014		
XylD (1.3 U/g)	None	1.69					
FAE-III (1.3 U/g) + *T. viride* xylanase (200 U/g)	None	26.3			0.14		
XylD (1.3 U/g) + *T. viride* xylanase (200 U/g)	None	27.6			0.15		
T. viride xylanase (200 U/g)	FAE-III (1.3 U/g)	28.5			0.11		
T. viride xylanase (200 U/g)	XylD (1.3 U/g)	29.3			0.15		
T. viride xylanase (200 U/g)	NaOH (1.0 M)	28.2	TR		0.23	0.39	0.18

[a] Data are means of duplicate analyses and are expressed as μmoles of compound released per gram of starting material. TR, trace.

TABLE 3. Influence of xylanases on the release of FA and FA dehydrodimers from destarched wheat bran by FAE-III

First treatment (concn [U/g])	Subsequent treatment of solubilized material (concn)	Release[a] of:					
		FA	8-8′ArylD	5-8′diFA	5-5′diFA	8-*O*-4′diFA	5-8′BenDi
FAE-III (1.3) + xylanase I (200)		10.3	—	—	TR	—	—
FAE-III (1.3) + XylA (200)		25.1	—	—	0.15	—	—
Xylanase I (200)	FAE-III (1.3 U/g)	11.2	—	—	TR	—	—
XylA (200)	FAE-III (1.3 U/g)	25.9	—	—	0.16	—	—
Xylanase I (200)	NaOH (1.0 M)	11.0	—	—	0.17	—	—
XylA (200)	NaOH (1.0 M)	25.3	—	—	0.15	0.25	0.041

[a] Data are means of duplicate analyses and are expressed as μmoles of compound released per gram of starting material. —, none detected; TR, trace.

lyzed supernatant contained FA and the dimers 8-8′ArylD, 5-8′diFA, 5-5′diFA, 8-*O*-4′diFA, and 5-8′BenDi (Tables 1 and 2), although the amounts were smaller than those produced by alkali treatment of destarched wheat bran directly. The fact that the esterases released only FA and 5-5′diFA from these xylanase-solubilized materials indicates that FAE-III and XylD are specific for the 5-5′ FA dehydrodimer esters and do not hydrolyze the other dimer esters, even though some occur at higher concentrations in the solubilized fraction of the cell wall.

We determined whether the source and type of xylanase affect the solubilization of esterified dimers. Xylanase I (11) and XylA (10) were used instead of the *T. viride* xylanase. Again, 5-5′diFA was the only dimer solubilized from wheat bran by FAE-III (1.3 U/g) in the presence of xylanase I (200 U/g) or XylA (200 U/g) (Table 3). As expected (1), the release of (mono)FA by FAE-III was higher in the presence of *T. viride* xylanase than in the presence of XylA, and xylanase I led to the lowest release of FA by FAE-III (Tables 2 and 3). There were only small differences between xylanases in the amount of 5-5′diFA released from wheat bran by FAE-III, but there were markedly different profiles of FA dimers (Tables 2 and 3). This indicates that the release of particular dimers depends on the nature of the xylanase and that xylanase I is much poorer at solubilizing cell wall material containing 8-*O*-4′diFA and 5-8′BenDi than xylanase I or XylA. Irrespective of the nature of the solubilized material, subsequent esterase treatment yields only the 5-5′diFA dimer.

DISCUSSION

FA dimers have been found in a wide range of *Graminaceae*. Ryegrass, barley straw, red clover, maize, and sorghum all contain alkali-extractable dehydro-FA dimers and truxillic acids. Barley cell walls contain six isomeric forms of the 5-5′ dimer (21). Suspension-cultured corn, cocksfoot, and switchgrass contained five dimers, including 5-5′diFA, 8-*O*-4′diFA, and 5-8′diFA (18). The presence of dimers is not related to the presence of lignin, since both maize and wheat grains contain diFA but only wheat contains lignified material (19). A diferuloyl arabinoxylan hexasaccharide $(FAXX)_2$, containing 5-5′-linked diFA has been obtained by enzymatic hydroysis of bamboo shoot cell walls (13). These cross-links have a number of physical consequences: they affect the nutrional value of forage (9), increase the mechanical strength of the cell wall (17), and limit cell wall growth (14). It has been claimed that phenolic dimers are the "molecular equivalent of spot-welding a steel mesh frame" (12), which illustrates their potential impact on physical structure. From the results presented here, the cross-links certainly limit hydrolysis by xylanases, and this effect depends on the source of the xylanase. It should be noted that the dimers decrease the accessibility to all xylanases, since ~60% of the total (mono)feruloylated materials was released from wheat bran, but a much lower percentage of the total dimers were released, even by the *T. viride* xylanase.

Esterases showed the highest di-FAE activity on material that had been presolubilized by xylanases. Obviously, if no diferuloylated material was solubilized by the xylanase in the first step, then there would be no increased de-esterification by subsequent esterase action. However, of all of the solubilized material released by any of the xylanases, only 5-5′diFA was released by either of the esterases. This demonstrates that XylD and FAE-III are specific for the release of this dimer and are not active on other dimers (even those present at a higher concentration). This is the first description of enzymes which can hydrolyze any esters of phenolic dimers in plant cell walls.

It is not clear why FAE-III and XylD are active only on the 5,5′diFA dimer and do not release 100% of the alkali-extractable dimer. The observed specificity could arise for two reasons: substrate accessibility and enzyme specificity. (i) The physical properties of arabinoxylan structures surrounding a dimeric linkage could be different from the rest of the arabinoxylan, and this would limit accessibility to certain parts of the arabinoxylan. (ii) FAE-III is active only on methyl esters of certain hydroxycinnamic acids, which includes ferulic and sinapic esters but not *p*-coumaric or caffeic esters (6). This shows that only certain substitutions on the aromatic ring can be tolerated, and this gives specificity to this enzyme. In contract,

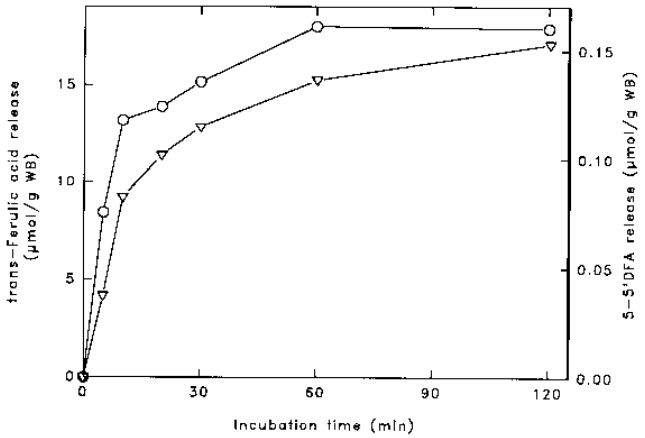

FIG. 4. Time course of release of FA (○) and the 5-5′ FA dehydrodimer (▽) from the soluble fraction of xylanase-treated, destarched wheat bran (WB). Wheat bran (0.4 g) was incubated with *T. viride* xylanase (200 U/g) for 7 h at 37°C in a final volume of 9 ml. After boiling (5 min) and centrifugation, the supernatant was removed and duplicate portions (0.45 ml of each) were incubated with FAE-III (0.013 U; 0.01 ml) for 0 to 2 h. Incubation mixtures were boiled and assayed for phenolic monomeric and dimeric acids as described in Materials and Methods. Alkali-extractable levels of FA and 5-5′diFA from the solubilized material were 282 and 1.98 nmol/sample, respectively.

XylD is active on all of these methyl hydroxycinnamate esters. Alternatively, the specificity may arise because of substitutions at the unsaturated bond, which may decrease or abolish enzyme activity. This point may be elucidated by chemical synthesis of soluble esters of the dimers.

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