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Hydrolysis of diethyl diferulates by a tannase from *Aspergillus oryzae*

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Abstract

Diferulic acid forms cross-links in naturally occurring plant cell wall polymers such as arabinoxylans and pectins. We have used model ethyl esterified substrates to find enzymes able to break these cross-links. A tannase from *Aspergillus oryzae* exhibited esterase activity on several synthetic ethyl esterified diferulates. The efficiency of this esterase activity on most diferulates is low compared to that of a cinnamoyl esterase, FAEA, from *Aspergillus niger*. Of the diferulate substrates assayed, tannase was most efficient at hydrolysing the first ester bond of the 5–5- type of dimer. Importantly and unlike the cinnamoyl esterase, tannase from *A. oryzae* is able to hydrolyse both ester bonds from the 8–5-benzofuran dimer, thus forming the corresponding free acid product. These results suggest that tannases may contribute to plant cell wall degradation by cleaving some of the cross-links existing between cell wall polymers.

Keywords: *Aspergillus oryzae*; cell wall polymers; Diethyl diferulates

1. Introduction

Micro-organisms need to produce a combination of enzymes, primarily carbohydrases and ‘esterases’ (able to remove side chain substituents) that act synergistically, in order to increase digestibility of the plant cell wall. Cross-linking of the cell wall polymers by ferulic acid dehydrodimers: 8–5-, 8–O–4-, 5–5- and 8–8- diFAs (Ralph, Quideau, Grabbber & Hatfield, 1994) (Fig. 1), is a major obstacle which limits the accessibility of main chain-degrading enzymes to the structural polysaccharides and reduces cell wall digestibility (Grabber, Hatfield & Ralph, 1998a,b). The breakage of one or both ester bonds from these dehydrodimer cross-links between plant cell wall polymers is essential for degradation of plant cell wall.

An esterase from *Aspergillus niger*, FAEA, is able to cleave both ester bonds from the 5–5-diferulate and the 8–O–4-diferulate and can release the corresponding free acids from xylanase solubilised plant material (Bartolome et al., 1997; Kroon, García-Conesa, Fillingham, Hazlewood & Williamson, 1999). However, this esterase was able to cleave only one ester bond from the 8–5-benzofuran type of dimer and, although this may be sufficient to facilitate the subsequent action of main polymers-degrading enzymes, it was not possible to release the free acid (García-Conesa et al., 1999). To date, there are no reports on the release of this dimer from plant material.

Microbial esterases are a broad group of enzymes able to hydrolyse the ester bond of a variety of naturally occurring esters of flavonoids and hydroxycinnamates present in plants. Definition and characterisation of these enzymes are based mainly on their specificity towards a range of synthetic and purified substrates. The availability of new substrates provides researchers with a tool to re-examine the specificity of esterases for hydrolysing the variety of components present in plant cell walls. For example, synthetic dehydrodiferulates (Ralph et al., 1994; Ralph, García-Conesa & Williamson, 1998) can be used to search for other esterases able to cleave plant cell wall cross-links, perhaps even more specific than FAEA from *A. niger*, and in particular, to search for esterases able to cleave the second ester bond from the 8–5-benzofuran diferulate, contributing to the digestion of the plant cell wall.

Tannin acyl hydrolases (EC 3.1.1.20), commonly referred to as tannases, are inducible enzymes produced by fungi, mainly by *Aspergillus* and *Penicillium* species (Lekha & Lonsanne, 1997) but they have also been described in yeast (Aoki, Shinke & Nishira, 1976), bacteria (Deschamps, Otuk & Lebeault, 1983; Nelson, Pell, Schofield & Zinder, 1995) and plants (Niehaus & Gross, 1997). Tannases produced by microorganisms probably serve as a mode of invasion into the host plant by hydrolysing tannins that are present in many herbaceous and woody plants (Lekha &

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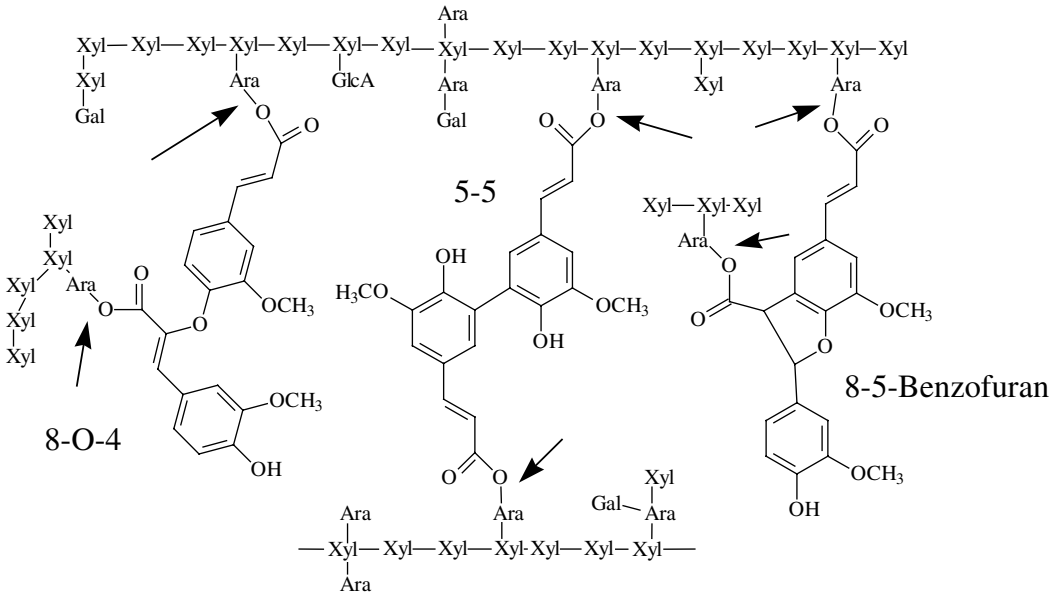

Fig. 1. Scheme showing the main diferulate cross-links described in plant cell walls (8–O–4-, 5–5- and 8–5-benzofuran diferulates).

Lonsanne, 1997). Tannases have been mostly characterised by their activity on these complex polyphenolics.

Tannases are esterases able to hydrolyse the 'ester' bond (galloyl ester of an alcohol moiety) and the 'depside' bond (galloyl ester of gallic acid) in substrates such as tannic acid, methylgallate and *m*-digallic acid (Lekha & Lonsanne, 1997). Two separate isoenzymes, tannase I and tannase II, with esterase and depsidase activity, respectively, have been described in *Aspergillus oryzae* (Beverini & Metche, 1990) but more recently, some pure tannases exhibiting both activities have been obtained (Barthomeuf, Regerat & Pourrat, 1994; Niehaus & Gross, 1997). Tannase has also been reported to act on substrates such as chlorogenic acid, (−)-epicatechin gallate and (−)-epigallocatechin-3-gallate (Lekha & Lonsanne, 1997). The activity of tannase is markedly inhibited in the presence of diisopropyl fluorophosphate suggesting that this enzyme is a typical serine esterase (Barthomeuf et al., 1994).

It is possible that the esterase and/or depsidase activity of tannases is also able to cleave other phenolics present in plant cell walls such as the dehydrodimer cross-links. In this report, the activity of a tannase from *A. oryzae* on a range of synthetic ethyl esterified diferulates is investigated. The results are compared to those obtained with another esterase, FAEA from *A. niger*, assayed under the same conditions.

2. Experimental

2.1. Enzymes

Pure tannase from *A. oryzae* was kindly provided by Novo Nordisk (Denmark). The cinnamoyl esterase (FAEA) was purified from culture supernatants of *A. niger* grown on oat spelt xylan according to a published procedure (Faulds & Williamson, 1994).

2.2. Substrates

Diethyl 8–5-benzofuran diferulate was synthesised using peroxidase-H_2O_2 and purified by flash chromatography (Ralph et al., 1998). Ethyl 8–5-benzofuran diferulate was obtained from controlled enzymatic hydrolysis of diethyl 8–5-benzofuran diferulate incubated with FAEA, and purification of the monoester product by reverse-phase preparative chromatography (García-Conesa, Plumb, Kroon, Wallace & Williamson, 1997). Diethyl 5–5-diferulate was prepared from acetylated divanillin (Richitzenhain, 1949). Diethyl 8–O–4-diferulate was synthesised according to the method of Ralph et al. (1994) and purified by reverse-phase preparative chromatography (García-Conesa et al., 1997). Epicatechin-3-gallate was a kind gift from Dr Alan Davies (Unilever, Colworth House, Sharnbrook, UK). Methyl ferulate was purchased from Apin Chemicals Ltd (Oxon, UK) and gallic acid (3,4,5-trihydroxybenzoic acid monohydrate) was obtained from Sigma (UK). All other chemicals were of AnalaR or HPLC-grade purity.

2.3. Protein determination

Protein concentration of an aqueous solution of pure tannase from *A. oryzae* was estimated using a commercial assay kit (Coomassie Plus, Pierce, Rockford, IL, USA) based on the method of Bradford (1976). Bovine serum albumin (0–25 μg/ml in water) was used as standard protein. Protein concentration of a solution of pure FAEA was calculated by determining its absorbance at 280 nm

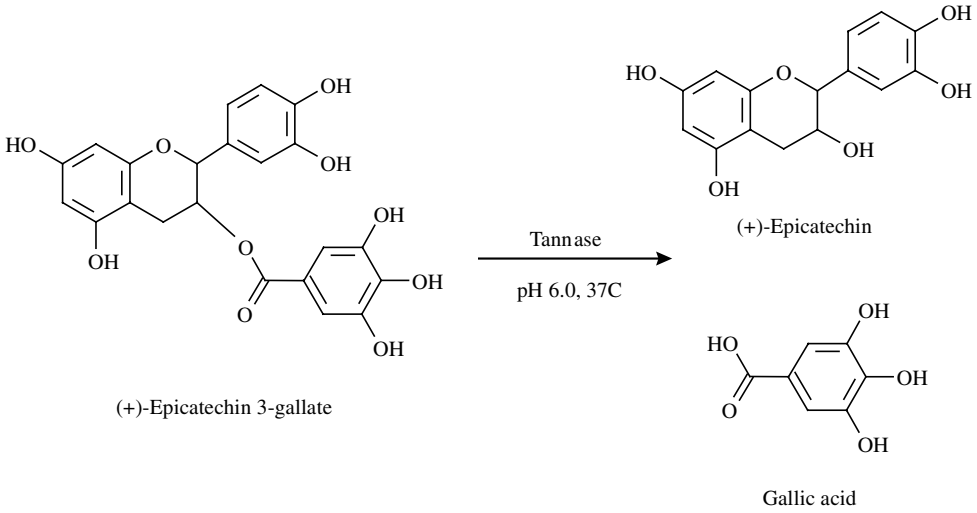

Fig. 2. Hydrolysis of (+)-epicatechin 3-gallate by tannase to form (+)-epicatechin and gallic acid.

(ϵ: 43,660 $M^{-1}\,cm^{-1}$, estimated on the basis of amino acid composition).

2.4. Esterase activity

Esterase activity was assayed as described by Faulds and Williamson (1991). Methyl ferulate (MFA; 1.0 mM final concentration) was prepared in Mops buffer (pH 6.0) and incubated with an appropriate amount of enzyme at 37°C. The reaction was terminated by the addition of acetic acid (pH $\leq$ 2.0). Release of free ferulic acid was measured by reverse-phase HPLC with detection at 325 and 280 nm (Waldron, Parr, Ng & Ralph, 1996), and quantified by reference to a ferulic acid calibration curve. All enzyme assays were performed in duplicate and with appropriate blanks to allow for correction for any background reactions. One unit (U) of activity was defined as the amount of enzyme releasing 1 μmol of ferulic acid per min at pH 6.0 and 37°C.

2.5. Tannase activity

Tannase activity was determined using (+)-epicatechin 3-gallate (ECG) as substrate, which is hydrolysed by the enzyme to form gallic acid and (+)-epicatechin (Fig. 2). (+)-epicatechin 3-gallate (1.8 mM final concentration) was prepared in Mops buffer (pH 6.0) and incubated with an appropriate amount of enzyme at 37°C. The hydrolysis process was terminated by boiling the reaction mixture for 10 min. Samples were filtered (0.2 μm) and injected (100 μl) onto a Prodigy ODS(3) reverse-phase column (25 cm × 4.6 mm i.d., 100 Å, Phenomenex, UK). The mobile phase included solvent A, consisting of tetrahydrofuran (THF) in water (2:98), and solvent B, acetonitrile. The flow rate was 1.0 ml/min. Separation was effected with gradient elution, starting at 0 min with 95% solvent A (5% solvent B) up to 7 min, decreasing to 0% solvent A (100% solvent B) at 10.0 min, held isocratically at 0% solvent A, 100% solvent B for a further 7 min, and followed by reconditioning the column.

Gallic acid was determined by HPLC/Diode Array with detection at 280 nm, where the absorbance of gallic acid shows its maximum, and was identified by spectroscopic analysis with diode array detection from 220 to 400 nm. Quantification was by integration of peak areas at 280 nm, with reference to a calibration curve. The response factor calculated for gallic acid at 280 nm was 26, 200 ± 1000 area units/ng; the standard curve was linear over the range: 50 to 3000 ng/100 μl. All enzyme assays were performed in duplicate and with appropriate blanks to allow for correction for any background reactions. One unit (U) of activity was defined as the amount of enzyme releasing 1 μmol of gallic acid per min at pH 6.0 and 37°C.

2.6. Activity on synthetic diferulates

The activity on esterified diferulates was assayed using a range of synthetic ethyl diferulates. Reaction mixtures contained the esterified substrate (final concentration: 0.2 mM, diethyl 5–5-diferulate; 0.3 mM, diethyl 8–O–4-diferulate; 0.05 mM, diethyl 8–5-benzofuran diferulate; 0.2 mM, ethyl 8–5-benzofuran diferulate), in 20% (v/v) DMSO in buffer (100 mM Mops, final pH 6.1). The activity was initiated by addition of the enzyme and incubations were performed at 30°C. Activity on the 5–5- and 8–5-diesters was measured after 2 h of incubation whereas the activity on the 8–5- monoester and the 8–O–4- diester was determined after 16 h of incubation. The reaction was terminated by the addition of acetic acid (final pH < 2.0) and samples were filtered (0.2 μm) prior to analysis by HPLC.

The release of product was monitored by HPLC/Diode Array using the method described by Waldron et al. (1996) with detection at 280 and 325 nm. The response factors for the reaction products, monoesters and free acids, were calculated carefully from appropriate solutions of purified

Table 1
Esterase and tannase activities for tannase from *A. oryzae* and FAEA from *A. niger* (activity values are expressed in nmol min^{-1} mg^{-1})

	Tannase (*A. oryzae*)	FAEA (*A. niger*)
Protein (mg/ml)	1.98	1.33
Activity on MFA	2.93	29400
Activity on (+)-epicatechin 3-gallate	26300	Nd[a]

[a] Nd: not detected.

synthetic compounds. All assays were performed in duplicate. Blanks containing the substrate plus the enzyme in 20% DMSO in buffer were incubated with acetic acid to correct for background peaks.

3. Results and discussion

3.1. Esterase and tannase activities

The activities on MFA and on ECG for the pure enzymes, tannase from *A. oryzae* and FAEA from *A. niger*, are shown in Table 1. Tannase exhibited a high activity on ECG (26.3 U/mg) and some esterase activity on the hydroxycinnamate substrate ($\sim 10^4$-fold less than FAEA). However, no gallic acid was formed when incubating the (+)-epicatechin 3-gallate with FAEA, even after incubation for 16 h with a large amount of pure enzyme (80 μg of pure FAEA). These results indicated that FAEA was not able to cleave the ester bond of the flavonoid molecule whereas tannase showed some ability to hydrolyse the hydroxycinnamate ester linkage in MFA.

3.2. Activity on synthetic esterified diferulates

HPLC analysis of reaction mixtures obtained after incubation of tannase with any of the four synthetic substrates tested indicated that, under the conditions of our assay, tannase from *A. oryzae* was active on the three ethyl-diesterified substrates and on the ethyl-esterified substrate, forming monoesters and free acid products. Tannase was able to cleave both ester bonds from the 5–5-diethyl diester forming two products that were identified as 5–5-monoester and 5–5-diferulic acid. The 8–O–4- diester was a very poor substrate for tannase and only minor quantities of the two possible monoesters products were detected. No free 8–O–4-diferulic acid was formed after 16 h of incubation. Tannase was also able to hydrolyse the 8–5-benzofuran diester forming predominantly a monoester product. However, two other small peaks were detected and identified on the basis of their retention time and spectra as a second monoester benzofuran product and as 8–5-benzofuran diferulic acid (Fig. 3). The formation of free 8–5-benzofuran diferulic acid was confirmed when the enzyme was incubated directly with the 8–5-benzofuran monoester (Fig. 4). These results indicated that tannase from *A. oryzae* was able to cleave both ester bonds from the benzofuran type of dimer releasing the free acid.

The ability of tannase to hydrolyse the diferulate compounds was compared to that of FAEA from *A. niger*, under the same assay conditions. The values obtained for both enzymes are presented in Table 2 and are expressed in

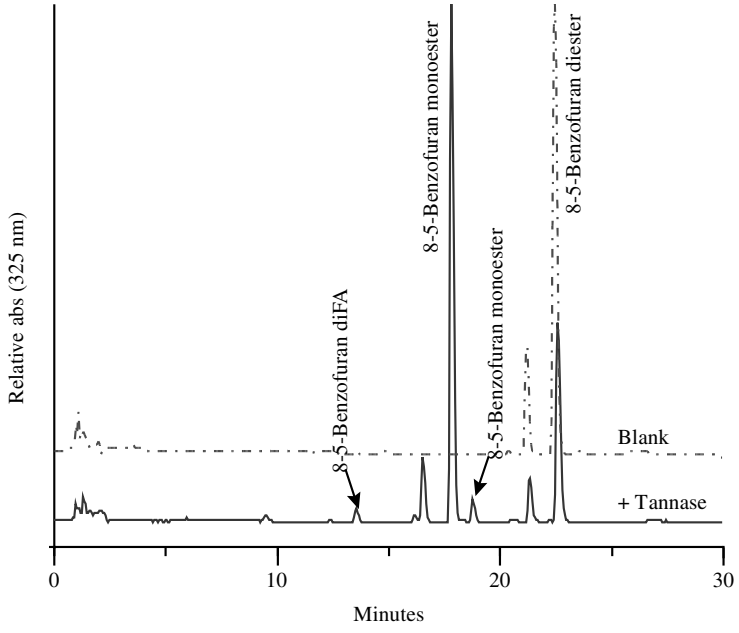

Fig. 3. HPLC elution profile at 325 nm of diethyl 8–5-benzofuran (diester substrate) and the products of its hydrolysis (monoester and free acid) by a tannase from *A. oryzae*.

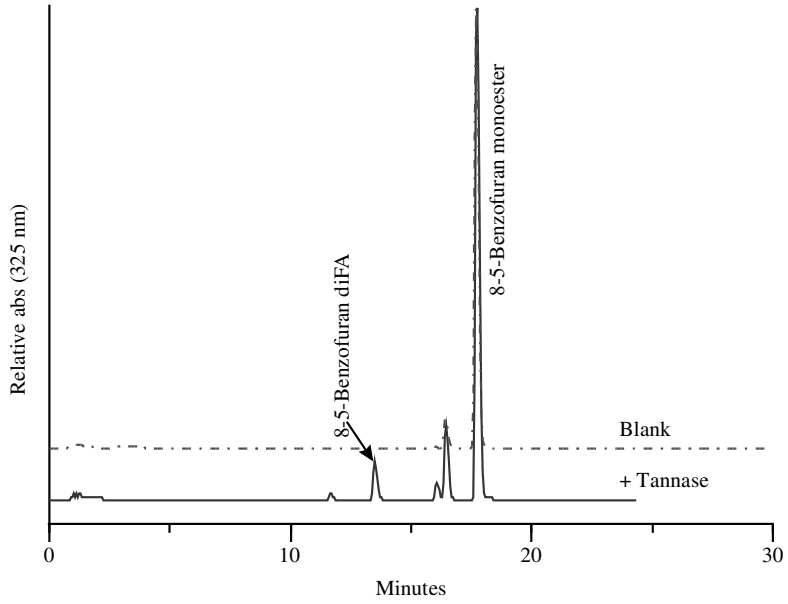

Fig. 4. HPLC elution profile at 325 nm of ethyl 8–5-benzofuran (monoester substrate) and the product of its hydrolysis (free acid) by a tannase from *A. oryzae*.

total nmol of product formed per mg of protein. As the incubation periods were very long, 2 h for the diethyl 5–5- and the diethyl 8–5-benzofuran diferulates and 16 h for the ethyl 8–5-benzofuran and the diethyl 8–O–4-diferulates, these values are not initial rates.

The results show that tannase from *A. oryzae*, is able to hydrolyse the ester bonds from the various synthetic diferulate substrates tested although the efficiency of this enzyme acting on the diferulates is much lower than that of FAEA from *A. niger*. This could be related to a poorer recognition of the hydroxycinnamate moiety by tannase. FAEA is an esterase much more highly specific for hydrolysis of some of the dehydrodiferulates cross-links. However, whilst FAEA is not active on the second ester bond from the 8–5-benzofuran type of dimer and did not form 8–5-benzofuran diferulic acid (even after an extended period of incubation with large amounts of enzyme) (García-Conesa et al., 1999), tannase was able to hydrolyse this second ester bond and formed some free acid (32 nmol of 8–5-benzofuran diferulic acid per mg of protein after 16 h of incubation).

Table 2
Hydrolysis of synthetic ethyl esterified diferulates by a tannase from *A. oryzae* and by FAEA from *A. niger*. Values are expressed in total nmol of product per mg of protein. Incubation of diethyl 5–5- and diethyl 8–5-benzofuran diferulates was for 2 h; incubation of ethyl 8–5-benzofuran and diethyl 8–O–4-diferulates was for 16 h

Substrate	Tannase (*A. oryzae*)	FAEA (*A. niger*)
Diethyl 5–5-diFA	166.0 (monoester)[a]	Nd[b] (monoester)
	12.0 (free acid)	≥ 2760 (free acid)
Diethyl 8–5-benzofuran diFA	34.0 (monoester)	≥ 1122 (monoester)
Ethyl 8–5-benzofuran diFA	32.0 (free acid)	Nf[c] (free acid)
Diethyl 8–O–4-diFA	15.0 (monoester)	5430 (monoester)
	11.0 (monoester)	2820 (monoester)
		870 (free acid)

[a] Product.
[b] Nd: not detected (under the described conditions, the monoester was all converted to free acid).
[c] Nf: not formed (even after extended period of incubation with large amounts of enzyme).

Tannases show high specificity for the phenolic site of the substrate. Substrates derived from benzoic acid carrying two *ortho*-hydroxyls seem to be better substrates than cinnamic acid derivatives (Niehaus & Gross, 1997; Scalbert, 1991). Esters with cinnamoyl residues, e.g. methyl cinnamates and sinapoyl glucose, were not accepted as substrates by a tannase purified from oak leaves and chlorogenic acid was hydrolysed at a very low rate (Niehaus & Gross, 1997). In the present report, a tannase with some activity on hydroxycinnamates derivative substrates is presented. Of the diferulate substrates assayed, tannase showed the best efficiency hydrolysing the first ester bond of the 5–5- type of dimer, followed by hydrolysis of one ester bond from the 8–5-benzofuran dimer, thus forming the corresponding monoester products. The 8–O–4- diester was a very poor substrate. The preferences shown by tannase are similar to those exhibited by FAEA but importantly and unlike the cinnamoyl esterase, tannase was able to recognise the second ester bond from the 8–5-benzofuran type of diferulate dimer.

4. Conclusions

Tannase from *A. oryzae* exhibits some esterase activity on several synthetic ethyl esterified diferulates. The efficiency of this activity is lower than that of other esterases, i.e. FAEA from *A. niger*. However, tannase from *A. oryzae* is able to hydrolyse the second ester bond from the 8–5-benzofuran dimer forming the corresponding free acid (Fig. 5). The

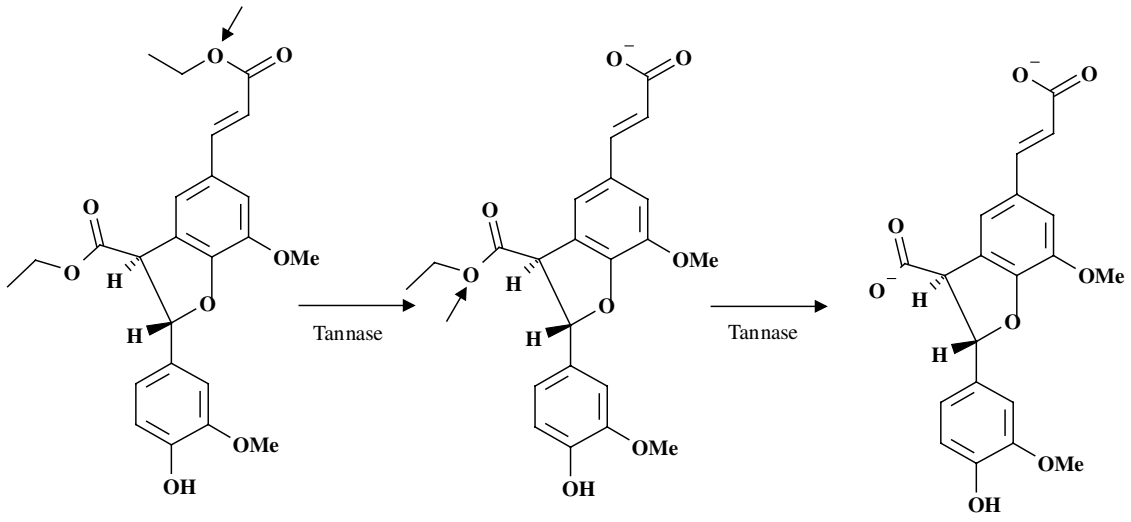

Fig. 5. Scheme showing the activity of tannase from *A. oryzae* on both ester bonds from the 8–5-benzofuran coupled diferulate.

results suggest that tannases may contribute to plant cell wall degradation by cleaving some of the cross-links existing between polymers.

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