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Feruloyl esterases as a tool for the release of phenolic compounds from agro-industrial by-products

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Abstract—Agro-industrial by-products are a potential source of added-value phenolic acids with promising applications in the food and pharmaceutical industries. Here two purified feruloyl esterases from *Aspergillus niger*, FAEA and FAEB were tested for their ability to release phenolic acids such as caffeic acid, *p*-coumaric acid and ferulic acid from coffee pulp, apple marc and wheat straw. Their hydrolysis activity was evaluated and compared with their action on maize bran and sugar beet pulp. The specificity of both enzymes against natural and synthetic substrates was evaluated; particular attention was paid to quinic esters and lignin monomers. The efficiency of both enzymes on model substrates was studied. We show the ability of these enzymes to hydrolyze quinic esters and ester linkages between phenolic acids and lignin monomer.
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1. Introduction

Plant cell walls are three-dimensional structures formed with a polysaccharide network of which cellulose, hemicelluloses and pectin are the most important components and it is well established that they contain hydroxycinnamic acids covalently linked to polysaccharides through ester linkages.[1] The role of hydroxycinnamates in the plant cell walls has been widely studied. They have been implicated in the regulation of cellular expansion and plant defense and they may reduce the digestibility of cell wall by restricting accessibility to carbohydrates. They are found in numerous plants and in significant quantities in agro-industrial derived by-products. Ferulic acid is abundant in wheat bran (0.5% w/w), sugar beet pulp (0.8% w/w) and maize bran (around 3.0% w/w).[2–4] It is linked to different positions on the arabinose sugar in wheat bran and sugar beet.[5,6] Moreover, cross-links through diferulic bridges are found both in heteroxylans and pectin tissues thus playing an important role in the structure of non-lignified cell walls.[4] *p*-Coumaric acid was found in significant amounts in maize bran (0.33% w/w).[4] Hydroxycinnamic acids occur widely in the cell walls of lignified plants such as cereal straws where they are linked through ester and ether bridges to polysaccharides and/or lignin.[7] Wheat straw contains, respectively, 1.24% and 0.66% of ferulic and *p*-coumaric acids. In plant material, hydroxycinnamic acids are also found as soluble conjugates of quinic acid named chlorogenic acids.[8,9] The commonest chlorogenic acid is 5-*O*-caffeoyl quinic acid. It is present in particularly high concentrations in numerous food and beverages such as coffee, pears,

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potato tubers and apple, and as a consequence in derived by-products. Analysis of the phenolic compounds in coffee pulp (the solid residue from coffee processing) indicated that chlorogenic acid was the main constituent (around 40%).[10] Apples have also been extensively studied. Values of 200 mg/kg total chlorogenic acid are reported but composition varies markedly with variety, cider varieties being richer than culinary.[11] The cider industry generates a residue that contains at least 500 mg/kg 5-*O*-caffeoyl quinic acid. The industrial use of hydroxycinnamates has attracted growing interest for several years since they and their conjugates were shown to be bioactive molecules, possessing potential antioxidant activities and health benefits.[12] The removal of these phenolic compounds and the breakdown of the ester linkages between polymers allows numerous exploitation for industrial and food applications.

Feruloyl esterases (EC 3.1.1.73), members of the carboxylic ester hydrolases sub-class of enzymes, have been found to cleave the ester linkage between hydroxycinnamic acids and sugars. These enzymes have been purified and characterized from a wide range of microorganisms, including bacteria and fungi (*Pseudomonas fluorescens*, *Penicillium funiculosum*, *Talaromyces stipitatus*, *Aspergillus niger*).[13–17] Recently, they were organized into four functional classes termed types A, B, C and D, which take into account substrate specificities against synthetic methyl esters of hydroxycinnamic acids, growth substrate requirements of the microorganisms and protein sequence identity.[18] Each feruloyl esterase has its own specificity with regard to the release of specific cinnamic acids. Two major enzymes were purified from *A. niger*, FAEA and FAEB (CinnAE), which are today classified as types A and C, respectively. Type C feruloyl esterases hydrolyze the four methyl esters of hydroxycinnamic acids generally used as model substrates (methyl ferulate, methyl sinapinate, methyl *p*-coumarate and methyl caffeate) but not diferulic compounds. In comparison, type A feruloyl esterases do not hydrolyze methyl ester of caffeic acid but are able to release diferulic compounds. These enzymes have both been overproduced in *A. niger*.[19,20] Studies of the ability of feruloyl esterases to hydrolyze natural substrates have mainly focused on FAEA and the release of ferulic acid. Different materials have been used such as sugar beet pulp, maize bran, wheat bran or oat hulls for the most important.[16,17,21–23] The pure enzymes are generally very slightly active when used directly on the raw substrate. An alternative strategy is to use suitable enzyme mixtures, including carbohydrate hydrolases and pretreatment of the raw material resulting in partial degradation of the more complex structures, as already reported in the release of ferulic acid from maize bran.[24] Besides, only a few studies have focused on the ability of feruloyl esterases to release *p*-coumaric and caffeic acids from natural substrates.

Our objectives in this study were to examine the different roles of two purified feruloyl esterases from *A. niger* FAEA and FAEB in the release of phenolic acids such as caffeic, ferulic and *p*-coumaric acid from natural agro-industrial residues. In order to better understand the mechanism of action of the two enzymes, we enlarged the panel of substrates. Apple marc and coffee pulp were chosen for their high content of caffeic acid and *p*-coumaric acid; in parallel, maize bran and sugar beet pulp were used for their content in ferulic acid. Special attention was also paid to wheat straw, which is typified by the presence of ferulic and *p*-coumaric acids linked to polysaccharides and/or lignin. In parallel, specificity against synthetic and model substrates was also determined.

2. Results and discussion

2.1. Hydrolysis of coffee pulp and apple marc

Coffee pulp and apple marc were selected for their high content of chlorogenic acids such as 5-*O*-caffeoyl quinic acid. Total amounts of caffeic, *p*-coumaric and ferulic acids were first determined after alkaline hydrolysis and HPLC analysis (Table 1). Coffee pulp and apple marc contained, respectively, 2.66 and 0.33 mg/g caffeic acid, 0.08 and 0.27 mg/g *p*-coumaric acid and 0.24 and 0.18 mg/g ferulic acid. Coffee pulp was rich in caffeic acid and ferulic acid, while apple marc contained greater amounts of *p*-coumaric acid. Initial chlorogenic acid content of both by-products was not visualized by this method since it was immediately hydrolyzed in caffeic acid and quinic acid. Before the action of the enzymes, no free phenolic acids could be detected. After incubation of both by-products with purified feruloyl esterases FAEA and FAEB, significant amounts of phenolic acids were released as shown in Figure 1. Only FAEB was active in the release of *p*-coumaric and caffeic acids from coffee pulp and apple marc. One hundred per cent of the alkali-extractable caffeic acid in the case of coffee pulp, and 83% in the case of apple marc were freed. Also, 73% and 34% of total *p*-coumaric acid was released. In comparison, FAEA had no significant effect. These results are consistent with the respective specificities

Table 1. Phenolic acid composition of agro-industrial by-products

Material	Caffeic acid	*p*-Coumaric acid	Ferulic acid
Coffee pulp	2.66	0.08	0.24
Apple marc	0.33	0.27	0.18
Steam-exploded wheat straw	0	2.13	1.35
Autoclaved maize bran	0	3.12	31.22
Sugar beet pulp	0	0	6.4

Values were determined after alkaline hydrolysis and expressed as mg/g dry material. Data are means of triplicate analysis.

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