論　文（Original Article）

Feruloyl Esterases from Suspension-cultured Rice Cells

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Abstract

Suspension-cultured rice cells produced at least two types of feruloyl esterase (FEase). The enzyme activity was assayed with methyl-ferulate as the substrate. FEase activity in the NaCl-extracted fraction became higher than the intracellular one at the late stage of cell growth though a lot of FEase activity in the intracellular fraction appared at the early stage of the cell growth. Rice FEases could release ferulic acid from methyl-ferulate and feruloyl arabinoxylan trisaccharide (FAXX). FEase in the NaCl extracted fraction at the late stage of cell growth showed higher activity toward FAXX than methyl-ferulate. However, intracellular FEase produced at the early stage of cell growth preferred methyl-ferulate over FAXX. The enzymes hardly attacked feruloyl arabinan, galactan, and xyloglucan oligosaccharides, and were not active on *p*-coumaroyl arabinoxylan trisaccharide and diferuloyl arabinoxylan hexasasaccharide. The results obtained here imply the involvment of FEase in cell wall modification.

Key words: *Oryza sativa*, feruloyl esterase, ferulic acid, cell wall

Introduction

Ferulic acid is one of the major hydroxycinnamic acid derivatives in plant cell walls, especially in the primary cell walls of Gramineae containing bamboo (Harris et al., 1976; Wende et al., 1997). It is well established that ferulic acid is linked to hemicellulosic polysaccharides in plant cell walls (Ishii, 1997). Ferulic acid plays an important physiological role in plant cell wall structure by cross-linking cell wall components through the formation of dehydrodimer of ferulic acids (Fry, 1986; Ralph et al., 1995; Iiyama et al., 1994). The isolation of diferuloyl arabinoxylan hexasaccharide, 5,5' -di-*O*-(diferul-9,9' -dioyl)-[α-L-Ara*f*-(1→3)-*O*-β-D-Xyl*p*-(1→4)-D-Xyl*p*] (diFAXX) from bamboo shoot cell walls directly proved the existence of cross-linking of arabinoxylan through diferuloyl bridges in plant cell walls (Ishii, 1991).

The content of ferulic and diferulic acids in plant cell walls was reported to correlate with mechanical properties of the cell wall (Kamisaka et al., 1990; Wakabayashi et al., 1997). Alkaline treatment of cell walls increased the ruminal microbial digestibility of gramineous forages since the phenolic constituents of cell walls including ferulic acid were liberated by alkali (Hartley et al., 1989). Many microorganisms secrete freruloyl esterase (FEase, EC 3.1.1.1), which removes the ferulic acid from feruloylated oligosaccharide (Williamson et al., 1998; Christov et al., 1993). FEases from microorganisms were thought to enhance the accessibility of glycosyl hydrolases such as xylanase by rupturing the ester bonds crosslinking cell wall polysaccharides (MacKenzie et al., 1987; Bartolomé et al., 1995). While some reports have shown the presence of FEase in plants (Niehaus et al., 1997; Sancho et al., 1999), its role is still to be determined.

In this report, we show the presence of FEases in suspension-cultured rice cells, and some enzymatic properties are characterized to estimate the role of rice FEase.

Materials and Methods

Substrates

Methyl-ferulate (Me-ferulate) was provided by Mr. S. Hishiyama of our institute. 5-5' Diferulate was kindly provided by Dr. N. Shibuya (National Institute of Agrobiological Resources). Feruloylated arabinoxylan trisaccharide [FAXX, {(F-Ara)-(1→3)-Xyl-(1→4)-(Xyl)}], feruloylated xyloglucan disaccharide [FXGlc, (F-Xyl)-(1→4)-Glc]], *p*-coumaroyl arabinoxylan trisaccharide [*p*CAXX, (*p*CA-Ara)-(1→3)-Xyl-(1→4)-Xyl]], and diFAXX were isolated from Driselase digests of bamboo shoot cell walls (Ishii et al., 1990; Ishii et al., 1990; Ishii, 1991). Feruloylated arabinan disaccharide [FAA, {(F-Ara)-(1→5)-Ara}, feruloylated arabinan trisaccharide [FAAA, {Ara-(1→3)-(F-Ara)-(1→5)-Ara}], and feruloylated galactan disaccharide

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[FGalGal, (F-Gal)-(1→4)-Gal]], were prepared from spinach leaves (Ishii et al., 1993; Ishii, 1994).

Cell culture and cell wall preparation

Suspension-cultured rice cells of *O. sativa* L. cv. nipponbare were kindly provided by Dr. N. Shibuya. They were maintained and subcultured every 7 days in a modified N-6 medium (Kuchitsu et al., 1993) on a reciprocal shaker at 24 ℃. To exclude the large aggregates, cells were filtered through a 20 mesh screen every week. Suspension-cultured cells were harvested at the desired time by filtration and washed with 0.1 M K-Pi buffer (pH 6.0). Washed cells were then ground to a fine powder under liquid nitrogen, and the cell wall was isolated as alcohol insoluble residue (York et al., 1985).

Hydroxycinnamic acid derivatives content determination

Ferulic acid, *p*-coumaric acid and 5-5' diferulate in the cell wall were released by alkaline saponification as described by Bartolomé et al. (1997). The released hydroxycinnamic acid derivatives were extracted by ethyl acetate and dissolved in 30% MeOH (pH 3.0, adjusted by diluted TFA). Samples were analyzed using HPLC (Shimadzu LC-10A system; Shimadzu, Kyoto) equipped with a Cosmosil 5C18-AR column (6 x 150 mm; Nacalai tesque, Kyoto) at 40 ℃ with a linear gradient of MeOH (30-50%, pH 3.0) at a flow rate of 1.0 ml $min.^{-1}$ Hydroxycinnamic acid derivatives in the effluents were detected and quantified by monitoring the peak areas at 310 nm.

Enzyme extraction

Washed cells were homogenized in an equal volume of extraction buffer (0.1 M K-Pi buffer, pH 6.0, containing 1 mM EDTA, 1 mM DTT, and 0.4 M sucrose) with quartz sand. The homogenate was then centrifuged at 15,000*g* for 15 min.

i) Preparation of the microsome fraction (Fr. I)

After centrifugation, the supernatant was recentrifuged at 100,000*g* for 30 min. The resultant pellet was washed with 0.1 M K-Pi buffer (pH 6.0) containing 1 mM DTT (buffer A). The precipitation resuspended in buffer A was used as Fr. I.

ii) Preparation of the intracellular fraction (Fr. II)

The supernatant obtained from centrifugation at 100,000*g* was collected, then dialyzed against buffer A. The dialyzate was considered Fr. II.

iii) Preparation of the NaCl-extracted fraction (Fr. III)

After centrifugation of the cell homogenate, the precipitation was extracted with two volumes of the extraction buffer containing 1 M NaCl for 30 min at 4 ℃. The NaCl extract was centrifuged at 15,000*g* for 15 min and the supernatant was collected, and dialyzed against buffer A. The dialyzate was termed the NaCl-extracted fraction, Fr. III.

iv) Preparation of the Driselase digested fraction (Fr. IV)

The NaCl-extracted precipitate was washed twice in an ice-cold extraction buffer. Bound enzymes were recovered from cell walls by treatment with a mixture of 0.25% (w v^{-1}) Driselase (Kyowa Hakko Kogyo Co., LTD., Tokyo) and 0.1% (w v^{-1}) Pectinase SS KYOWA (Shin Nippon Kagaku Kogyo Co., Ltd., Angyo) in 0.1 M K-Pi buffer (pH 6.0) at 25 ℃ with shaking. Driselase and Pectinase SS contained no FEase activities. After 20 hr of reaction and subsequent centrifugation at 15,000*g* for 15 min, the supernatant was dialyzed against buffer A. The dialyzate was designated Fr. IV.

Feruloyl esterase assay

FEase activity was assayed with Me-ferulate as substrate. The reaction was initiated by adding 25 μl of enzyme solution to 25 μl of 2 mM Me-ferulate solution. The enzyme reaction was carried out at 35 ℃ for 20 min. The reaction was then stopped by heating at 100 ℃ for 5 min after the addition of 50 μl of 0.1 M acetic acid. The resultant reaction mixture was filtered through a 0.45 μm membrane (Millipore, Freehold) and aliquots (20 μl) of the filtrate were applied to a Cosmosil 5C18-AR column. Enzymatically released ferulic acid in the effluent was detected and quantified by monitoring the fluorescence at 330 nm (excitation) and 435 nm (emission). One unit of FEase activity was defined as the amount of enzyme required to liberate the equivalent of 1 mmol of ferulic acid per min.

Optimum pH and temperature of feruloyl esterase

The optimum pH for the enzyme activities was measured with Me-ferulate as substrate by the feruloyl esterase assay method described above but at various pHs. The following buffers were used: 50 mM K-Pi buffer (pH 6.0 to 7.5), 50 mM Tris-HCl buffer (pH 7.0 to 8.5). The optimum temperature of each FEase was measured by the feruloyl esterase assay method at various temperatures between 25 ℃ and 50 ℃.

Substrate specificity of feruloyl esterase

The enzyme activity against some feruloylated compounds and *p*CAXX was assayed as follows. The reaction mixture contained an equal volume of (2 mM) of the substrate solution and enzyme solution. The enzyme was dialyzed against 0.1 M K-Pi buffer (pH 7.5) containing 1 mM DTT before use. The enzyme reaction was carried out at 45 ℃ for 20 min because the optimum temperature of rice FEase was 45 ℃. Enzymatically released ferulic acid or *p*-coumaric acid was monitored

and quantified as described in the ferulic acid esterase assay method. The enzyme reaction was also done with diFAXX as the substrate. An equal volume of 0.2% diFAXX solution was added to the enzyme solution. The reaction products from diFAXX were analyzed on a TSK-gel G2500PW column (7.5 x 300 mm; Tosoh, Tokyo) at 40 ℃ with 30% MeCN (pH 3.0) as solvent at a flow rate of 0.6 ml $min.^{-1}$

Results and discussion

Hydroxycinnamic acid derivatives content in the cell wall

Changes in the content of hydroxycinnamic acid derivatives in the cell walls of suspension-cultured rice (*Oryza sativa* cv. nipponbare) cells during cell growth are shown in Fig.1. The cells rapidly increased their fresh cell weight after 4 days of cultivation. After an initial decline during the first 4 days, the amount of ferulic acid in the cell walls gradually increased according to the cell growth. The amount of *p*-coumaric acid and 5-5' diferulate in the cell wall slightly increased at the late stage of cell growth. Fujii et al. (1993) revealed that ferulic acid in the cell walls of bamboo (*Phyllostachys pubescens*) shoots was synthesized at the early stage of the bamboo shoot growth before lignin accumulation, while the content of *p*-coumaric acid increased with progress of lignification. The accumulation pattern of hydroxycinnamic compounds in suspension-cultured rice cells showed a similar pattern as that of bamboo shoots, indicating that significant structural changes in the cell walls may occur at the late stage of cell growth.

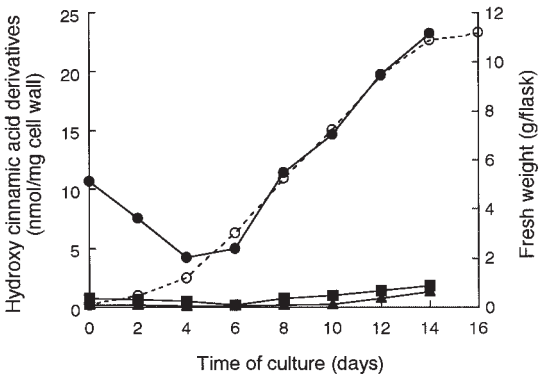

Fig.1. Changes in the content of hydroxycinnamic acid derivatives in the cell wall of suspension-cultured rice cells. ●, ferulic acid; ▲, *p*-coumaric acid; ■, 5-5' diferulate; ○, fresh cell weight. The results are expressed as the means of two independent experiments.

Feruloyl esterase activity

Changes in the amount of FEase activities measured with Me-ferulate as substrates are shown in Fig.2. We determined the amount of enzymatically released ferulic acid by their fluorescence. Although the fluorescent intensity of ferulic acid and feruloyl esters vary with pH (Harris et al., 1976), good quantification and sensitivity were obtained under assay conditions. FEases were detected in the intracellular fractions (Fr. IIs) and the NaCl-extracted fractions (Fr. IIIs). FEase activities in the microsome fractions (Fr. Is) were trace and no FEase activities were detected in the Driselase digested fractions (Fr. IVs). Fraction IIs contained considerable amounts of FEase activity during the early stages of cell growth but the activity gradually decreased thereafter. In contrast, FEase activities in Fr. IIIs showed little change during cell growth. FEase activity per mg protein from Fr. III prepared from 12-day cultured cells (Fr. 12-III) was about two times higher than that of 4-day cultured cells (Fr. 4-III).

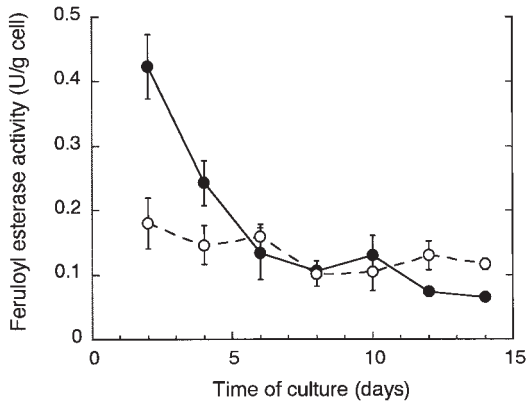

Fig.2. Changes in the amount of feruloyl esterase activities in the intracellular fraction and the NaCl-extracted fraction. ●, Intracellular fraction (Fr. II); ○, NaCl-extracted fraction (Fr. III). Mean values±SE (n = 3) are given.

Optimum pH and temperature of feruloyl esterases

The amount of FEase activity was more abundant in Fr. III than that in Fr. II at the late stage of cell growth. Some properties of FEases from Fr. 4-II (Fr. II prepared from 4-day cultured cells) and Fr. 12-III (Fr. III prepared from 12-day cultured cells) were examined to clarify the differences between these enzymes.

The optimum pH and temperature of Fr. 4-II FEase were 7.5 and 45 ℃, respectively. Similar results were obtained with Fr. 12-III FEase. The buffer composition had different effects on the two enzymes. Although Fr. 4-II and Fr. 12-III FEases exhibited highest activity in K-Pi buffer at pH 7.5, Fr. 12-III FEase showed the optimum pH to be 8.0 in the Tris-HCl buffer. Me-ferulate was stable under assay conditions.

Substrate specificities of feruloyl esterases

In previous studies, *K*m values of FEases for Me-ferulate were reported to range in concentration from 0.14 mM (*Penicillium pinophilium*) to 1.9 mM (*Streptomyces olivochromogenes*) (Williamson et al.,

1998). Substrate specificities of FEases from Fr. 4-II and Fr.12-III were therefore examined with 2 mM Me-ferulate and some feruloylated oligosaccharides as substrates (Fig.3). Fr. 4-II FEase showed higher activity with Me-ferulate than FAXX. While feruloylated arabinan disaccharide (FAA) was degraded, the enzyme hardly attacked FAAA, FGalGal, FXGlc, and *p*CAXX. The most susceptible substrates for Fr. 12-III FEase among those examined were FAXX followed closely by Me-ferulate. Fr. 12-III FEase hardly degraded FAAA, FAA, FGalGal, FXGlc or *p*CAXX. Both Fr. 4-II and Fr. 12-III FEases were inactive toward diFAXX (data not shown). In short, the rice FEases preferred Me-ferulate and FAXX as substrates.

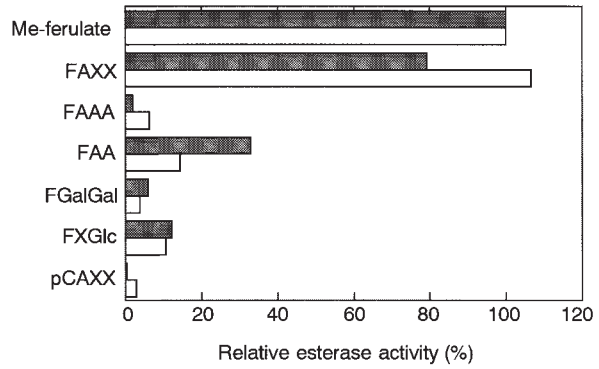

Fig.3. Analyses of the substrate specificity of feruloyl esterase. The rates of degradation for Me-ferulate and feruloylated oligosaccharides were measured for Fr. 4-II FEase (black bars) and Fr. 12-III FEase (white bars). The results were expressed as the means of two independent experiments. The activity against Me-ferulate was set to 100.

A series of feruloylated arabinoxylan fragments were detected in the enzymatic digest of *O. sativa* endosperm cell walls, though the isolation and structural characterization of feruloyl oligosaccharides were not accomplished (Shibuya, 1984; Shibuya et al., 1978; Wende et al., 1997). These reports verified the existence of feruloylated arabinoxylan oligosaccharides in the cell walls of suspension-cultured *O. sativa* cv. nipponbare. Though FXGlc and *p*CAXX were isolated from bamboo shoot cell walls, they were much less abundant than FAXX there (Ishii et al., 1990). As far as we know, feruloylation occurs on arabinoxylan and xyloglucan in monocots. In the cell walls of dicots, feruloyl group is attached to the arabinan, (1→4)-linked galactan and arabinoxylan (Ishii, 1997). However, there has been no report of feruloylated arabinan and (1→4)-linked galactan in gramineous monocots. The FEase from rice cells liberated ferulic acid from the feruloylated arabinoxylan fragment, which was assumed to be one of their cell wall components.

Several FEases have been isolated from microorganisms and their properties characterized (Williamson et al., 1998; Christov et al., 1993). FEases from *Aspergillus niger* (Ralet et al., 1994), *Neocallimastix* MC-2 (Borneman et al., 1992), and *S. olivochromogenes* (Faulds et al., 1991) hydrolysed Me-ferulate slower than FAXX. It would be natural for microbial FEase to prefer FAXX due to their role in breaking down the hemicellulosic component of plant cell walls (Williamson et al., 1998). Rice FEase from Fr. 12-III also preferred FAXX to Me-ferulate. Given the cell wall metabolism and cell wall restructure during plant growth, this substrate specificity of rice FEase from Fr. 12-III might be related to the regulatory role in the cell wall. In contrast, Fr. 4-II FEase preferred Me-ferulate rather than FAXX. While Me-ferulate is a convenient tool to examine the activity of FEase, it is a synthesized compound and not a natural substrate for FEase. The major role of FEase included in Fr. 4-II, which showed a high affinity for Me-ferulate, remains unknown.

Current results show that at least two kinds of FEase are present in suspension-cultured rice cells and their substrate specificities are different. One of these, produced at the late stage of cell growth, may be involved in cell wall modification in rice.

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イネ懸濁培養細胞のフェルロイルエステラーゼ

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要　旨

イネ懸濁培養細胞は、少なくとも２種類のフェルロイルエステラーゼ（FEase）を生産することが明らかになった。酵素活性は、メチルフェルレイトを基質として検出した。細胞におけるFEase活性は、細胞成長の初期には細胞内においてより多く発現されたが、後期には細胞壁抽出画分の活性の方が高くなった。イネ由来のこれらのFEaseは、メチルフェルレイトとフェルロイルアラビノキシロオリゴ糖からフェルラ酸を遊離させた。細胞壁から抽出されたFEaseでは、メチルフェルレイトよりもフェルロイルアラビノキシロオリゴ糖の方が適した基質であったが、細胞内のFEaseは、メチルフェルレイトの方をよく分解した。イネのFEaseは、フェルロイルアラビノオリゴ糖、フェルロイルガラクトオリゴ糖、フェルロイルキシロオリゴ糖、パラクマロイルアラビノキシロオリゴ糖及びジフェルロイルアラビノキシロオリゴ糖はほとんど分解しなかった。これらの結果は、植物細胞壁の変化にFEaseが関与していることを示唆するものである。

キーワード：イネ、フェルロイルエステラーゼ、フェルラ酸、細胞壁

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