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A NOVEL PROTEIN FROM MUNG BEAN HYPOCOTYL CELL WALLS WITH ACETYL ESTERASE ACTIVITY

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Key Word Index—*Vigna radiata*; Fabaceae; mung bean; acetyl esterase; cell wall; pectin; hypocotyl.

Abstract—An acetyl esterase was purified from cell walls isolated from mung bean hypocotyls. The purified enzyme had an apparent M_r of 43 300 and an apparent pI > 9. It rapidly deesterified triacetin and *p*-nitrophenylacetate and slowly released acetate from beet and flax pectins, the deesterification rate being increased by previous demethylation of the pectins. No significant peptide sequence identity between the acetyl esterase and any known protein could be found in protein data bases.

INTRODUCTION

Pectins are major components of all primary cell walls of dicotyledons. The backbone is a (1,4)-linked α-D-galacturonan chain interspersed with (1,2)-linked-α-L-rhamnose. A proportion of the galacturonyl residues is esterified. Most of them are methoxylated at C-6 but some are acetylated at C-3 [1]. Demethoxylation is generally supposed to occur *in muro* catalysed by cell wall pectin methyl esterases (PMEs) since (i) pectins are secreted from the Golgi with a very high methylation degree [2] and (ii) PMEs are widely distributed cell wall enzymes. The control of the degree of acetylation is still unknown, although the acetylation process is supposed to occur inside the Golgi via acetyl CoA [3], the acetylated pectins then being incorporated into the cell wall. Recently, pectin acetyl esterases have been isolated from orange peel [4] and from *Aspergillus aculeatus* [5], but no information was given concerning their localization. While investigating mung bean cell wall esterases [6], we observed previously that the cell walls contained not only several PMEs but also another esterase able to cleave the ester bond of *p*-nitrophenyl acetate (*p*NPA), but unable to remove the methoxyl group linked to C-6 of the galacturonosyl residues of *Citrus* pectin. Experiments were therefore undertaken in order to purify and characterize this cell wall acetyl esterase (AE).

RESULTS AND DISCUSSION

Purification

Cell walls were isolated from the upper part of mung bean hypocotyls and then incubated in 1 M NaCl, a treatment which solubilized the ionically-bound proteins. This protein fraction was able to release acetate from triacetin, which suggested the ocurrence of an AE. These data confirmed previous results showing that mung bean cell walls contain a protein able to release *p*-nitrophenol from *p*NPA [7]. As already observed using this compound as substrate [7], after chromatography on CM Sepharose CL 6B, about 80% of the AE activity was recovered in a single peak eluting with 0.28 M NaCl. Nevertheless, this fraction also exhibited significant PME activity. It was therefore applied to a column of MonoS equilibrated in 0.05 M Na phosphate buffer pH 7. PME

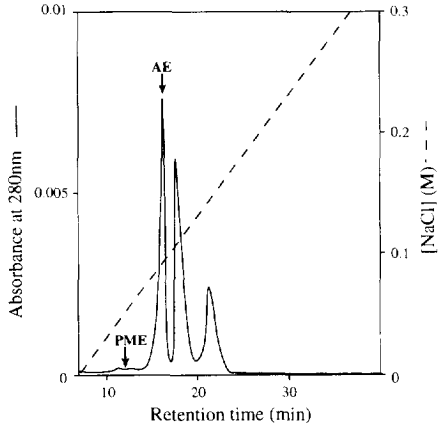

Fig. 1. Purification of AE by FPLC. The protein fraction eluted from CM Sepharose CL 6B with 0.28 M NaCl loaded on to a Pharmacia monoS column. Elution was performed with a linear 0–0.3 M Nacl gradient. PME and AE activities are indicated with arrows.

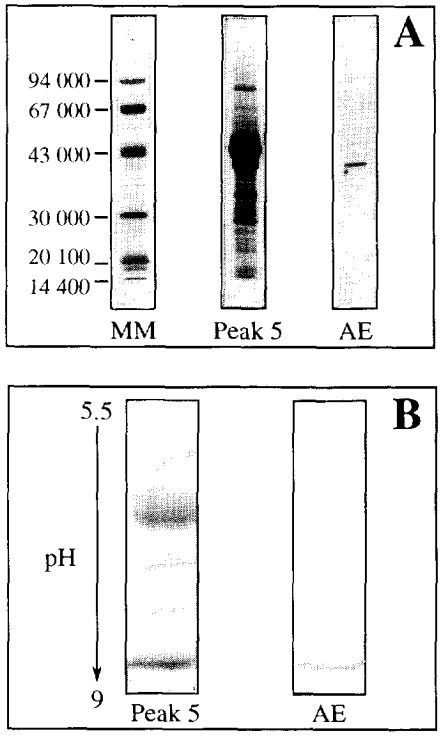

Fig. 2. Electrophoretic patterns of AE preparations before (lane 1) and after (lane 2) chromatography on MonoS. (A) SDS–PAGE (peak 5: 8 μg proteins; AE: 0.4 μg proteins); (B) IEF (peak 5: 16 μg proteins; AE: 8 μg proteins). Proteins silver stained. MM: molecular mass markers; peak 5: peak 5 after CM Sepharose; AE: purified acetyl esterase.

and AE activities eluted separately, the first one with 0.03 M NaCl, the second one with 0.09 M NaCl (Fig. 1). AE was recovered as a single protein band when submitted to SDS–PAGE or IEF (Figs 2A and 2B). Procedures carried out to purify the AE from mung bean hypocotyls are described in the Experimental. Yields, specific activities, recoveries and purification factors of AE are shown in Table 1. The relatively low values for the purification factors can be explained by (i) the low protein content of the cell wall extract resulting in a high specific activity in this fraction and (ii) the instability of AE activity which decreased during storage at − 15°, especially when the enzyme extract was submitted to successive freeze-thawings.

Properties

Physicochemical properties of the purified AE are summarized in Table 2. Activities were assayed using both *p*-nitrophenyl acetate and triacetin as substrates. Contrary to orange peel AE, the mung bean enzyme exhibited an alkaline pI and its optimal pH with both substrates was less acidic than the values reported for orange or *Aspergillus* AE. As already reported for orange AE [4], K_m values were relatively high, indicating a low affinity, especially towards triacetin. In this case, the AE may have more difficulties gaining access to this substrate, because of the steric disposition of the three ester linkages, whereas in the case of *p*NPA, the aromatic ring presents only one ester bond. This might explain the difference in the affinity towards these two substrates. AE was also incubated with pectins extracted from beet, apple and flax (Table 3), the acetylation degrees of which were, respectively, *ca* 30%, 5% and 30%, with the degree of methylation being *ca* 75% for both beet and apple pectins, flax pectin exhibiting very few methyl groups [8–10]. Release of acetate could only be measured after long incubation times (20 hr), probably because pectins are complex molecules, where ester bonds might be less accessible to AE than ester bonds of *p*NPA or triacetin. Significant deacetylation occurred only with flax pectin which is both highly acetylated and lightly methylated. Beet and apple pectins which presented relatively high methylation degrees, were pre-treated with the PME isoform PE_3 extracted from mung bean hypocotyl cell walls [6], the enzyme being then inactivated (100°, 5 min) and the demethoxylated pectins treated with AE. The rate of reaction was increased 25-fold when compared to untreated beet pectin and only 2.7-fold when compared to untreated apple pectin. These data showed that mung bean AE, like orange AE, is more active on galacturonyl residues which are not methoxylated. Moreover, these results suggest that along the galacturonan chains, methyl and acetyl groups are randomly distributed, acetylated galacturonic acids interspersing the highly methylated backbone. The low activity measured with apple pectin might be due to the low acetyl content of this

Table 1. Enzyme yields during purification of AE from mung bean hypocotyl cell walls (activity refers to the release of acetate from triacetin)

Purification step	Yield: Protein (mg)	Yield: Activity (nkat)	Specific activity (nkat mg^{-1} protein)	Recovery (%)	Purification factor
Crude homogenate	90	1933	21.5	100	1
CM Sepharose (peak 5)	15	1506	100	78	4.7
MonoS HR 5/5	2.6	330	127	17	5.9

Table 2. Physicochemical parameters for mung bean AE

Parameter	Comment	Value
M_r	SDS–PAGE	43 300
K_m	triacetin	73 mM
	p-nitrophenylacetate	3.3×10^{-3} M
V_{max}	triacetin	106 nkat mg^{-1}
	p-nitrophenylacetate	25 nkat mg^{-1}
pH optimum	triacetin	6.0
	p-nitrophenylacetate	6.5
Apparent pI	IEF	> 9

Table 3. Effect of substrate on acetyl release

Substrate	Pretreatment	Enzyme activity (nkat mg^{-1} protein)
Beet pectin	None	1.6
	Demethylation with PME	41
Apple pectin	None	0.9
	Demethylation with PME	2.4
Flax pectin	None	8.8

polysaccharide. It can also be noted that AE released more acetate from demethylated beet pectin than from flax pectin. However, the ratio of galacturonic acid to neutral sugars was higher in beet (*ca* 3:2) [8] than in flax (*ca* 1:3) pectin. In both cases, AE was incubated with 1% pectin, which represents 0.6% galacturonic acid for beet pectin, but only 0.25% galacturonic acid for flax pectin. Therefore, less substrate was available with flax pectin, which could explain the lower AE activity. Moreover, the neutral branches may hinder the accessibility of the substrate. Finally, as reported for orange AE [4], and contrary to *Aspergillus* AE [5], the mung bean AE was much more active on triacetin than on pectins, whatever their origin.

Heat stability of mung bean AE was also investigated. The enzyme was inactivated quite rapidly at temperatures above 50°; for example, enzyme activity was reduced by 60% after 5 min at 60°. It could be noted that the purified enzyme lost *ca* 35% of its maximal activity after 8 min incubation at 50°, whereas the AE activity of the crude cell wall extract was enhanced by such a treatment, but decreased rapidly at 55°. In this case, the stimulation induced by the 50° pretreatment might have resulted from destruction during this pretreatment of a thermosensitive compound which inhibited the AE activity, this compound being lost during the purification procedure.

Isolation and sequencing of acetyl esterase peptide fragments

Figure 3 shows the peptide map provided by the RPLC separation of the tryptic peptides and the partial sequences obtained with four of them and the *N*-terminus of the entire AE. The peptide T2 revealed two sequences (named T2 and T2'), which could be determined because of the 1:10 ratio of the two peptides.

The search for homologies in protein data bases failed to reveal any significant sequence identity of the AE with

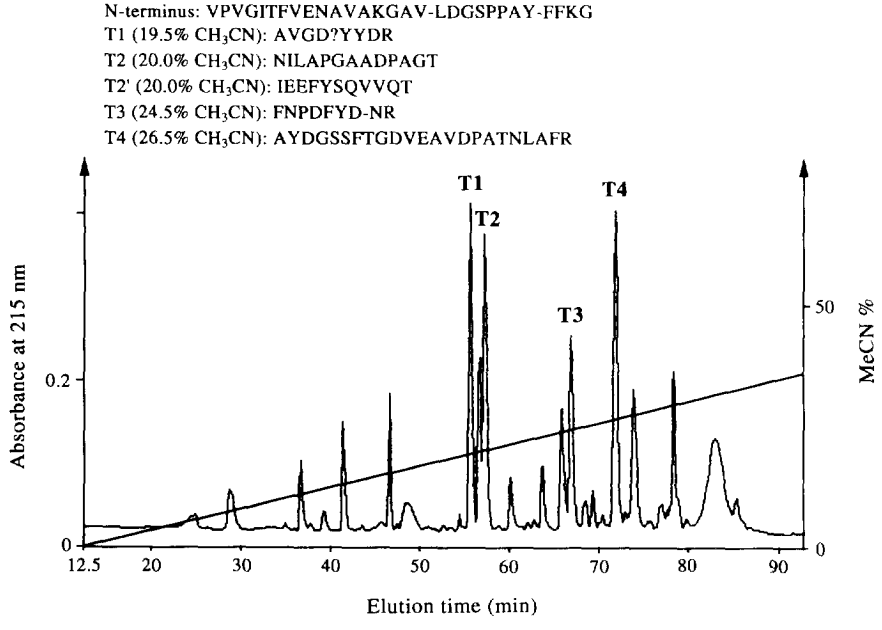

Fig. 3. Reverse-phase HPLC chromatograms of peptides resulting from acetyl esterase tryptic cleavage (peptide map) and partial sequence. Except for the *N*-terminus, the sequences correspond to those of the peptides obtained after trypsinolysis numbered according to the RPLC profile. The straight line represents the MeCN gradient.

any known protein, showing that the mung bean AE is a novel protein without connection with any other cell wall enzyme, such as PME.

In conclusion, the above reported data reveal the occurrence in mung bean hypocotyl cell walls of a novel protein, active on various ester bonds. The sequencing of its genomic sequence is now under way, in parallel with those of specific cell wall esterases only able to cleave the ester bond linking methanol to galacturonyl residues. The sequestration inside the cell wall of an unspecific esterase activity might be considered as a detoxification process, exocytosis clearing the cytoplasm from enzymes, the activity of which could damage important biological molecules. However, a possible role in the *in muro* regulation of degree of acetylation of the pectin cannot be excluded, but further research is needed in order to elucidate the exact functions of this new cell wall protein.

EXPERIMENTAL

Biological materials. According to a previously described procedure [6], seeds of *Vigna radiata* (L.) Wilczek were soaked in tap H_2O for 2 hr, placed on moist vermiculite and covered with a wet cloth. After 3 days at 26° in the dark, seedlings with hypocotyls 45 mm (± 5 mm) long were selected; 2.5 cm-long segments were excised below the hook. About 400 g of hypocotyls were ground in 2 mM Na-K-Pi buffer, pH 6, with a Sorvall omnimixer and filtered through cheese-cloth. The insoluble fr. was resuspended in the same buffer, incubated in 0.1% Triton, exhaustively washed with H_2O and then suspended in distilled H_2O. Around 4.5 g cell walls (dry wt) were obtained.

Citrus pectin, *p*NPA and triacetin were purchased from Sigma. Sugar beet pectin was a gift from Dr J. F. Thibault (INRA, Nantes), apple pectin a gift from Dr A. Baron (INRA, Rennes) and flax pectin a gift from Dr C. Morvan (University of Rouen).

Extraction and purification of AE. All steps were conducted at 4°. The enzymes were extracted (×2) from isolated cell walls with 0.05 M Na succinate buffer (pH 5) plus 1 M NaCl. The extract was reduced to a smaller vol. by ultrafiltration on an Amicon PM 10 membrane and its pH adjusted to 6 with 0.01 M Na-K-Pi buffer. This crude cell wall extract was then submitted to cation-exchange chromatography on CM Sepharose CL 6B (30 × 1.5 cm; flow rate 25 ml hr^{-1}). The column was first equilibrated with 0.01 M Pi buffer, pH 6, and elution was carried out with a linear gradient from 0–0.3 M NaCl. Frs of 5 ml were collected and their AE activity estimated from their ability to hydrolyse *p*NPA [7]. Active frs were pooled, concd and desalted by ultrafiltration. The most active fr. (peak 5) was then submitted to cation-exchange FPLC on a monoS HR 5/5 Pharmacia column (5 × 0.5 cm; flow rate 1 ml min^{-1}), elution being carried out with a linear 0–0.3 M NaCl gradient, established over 30 min. Frs (1 ml) were collected and assayed for their PME and AE activities.

Enzymatic assays. AE activity was measured using *p*NPA and triacetin. Hydrolysis of *p*NPA was estimated spectrophotometrically at 400 nm by the formation of *p*-nitrophenol. The assay mixt. (3 ml) contained 0.1 M Na-K-Pi buffer, pH 7 and 2 mM *p*NPA. Hydrolysis of triacetin was estimated by measuring the release of acetate. Triacetin (100 mM) was first incubated with enzyme at 30° in 0.025 M citrate-Pi buffer, pH 6, for 10 min. After immersion in H_2O at 100° for 2 min, the sample was cooled and the concn of acetate measured using an enzyme-linked kit from Boehringer according to the manufacturer's instructions. For measurement of pectin AE activity, the samples were treated as follows: methylated or demethylated pectin (1%) was incubated with enzyme for 20 hr at 30° in 0.025 M citrate-Pi buffer, pH 6. The reaction was stopped and acetate concns measured using the Boehringer kit. Pectin was demethylated by incubation with mung bean PME isoform A3 [6] (300 nkat ml^{-1}, 30 min, 30°), and then transferred at 100° for 5 min to inactivate the enzyme. PME activity was assayed either titrimetrically or qualitatively, using *Citrus* pectin as substrate, as previously described [6]. Protein concns were determined according to the micromethod of Bradford [11] using a Biorad kit and BSA as standard.

Electrophoresis. SDS–PAGE was performed on unidimensional 12.5% gels, according to ref. [12], using a Phast-System electrophoresis unit (Pharmacia-LKB). IEF was performed on ultrathin (0.5 mm) polyacrylamide slab gels (10% acrylamide, 0.26% *bis*acrylamide), containing 10% Pharmacia 3–10 pH range ampholines. The IEF was run on an LKB Multiphor 2117, using an LKB 2103 power supply at 30 W for 1 hr; NaOH (1 M) and H_2SO_4 (1 M) were used as catholyte and anolyte, respectively. Proteins were visualized by silver staining using a Biorad kit, according to the manufacturers's instructions.

Desalting of AE. AE was adsorbed by centrifugation on PVDF membrane according to ref. [13] using Prospin filters (Applied Biosystems Inc.).

Tryptic digests of AE and isolation of digested peptides. AE was adsorbed onto PVDF membrane prior to trypsinolysis performed according to ref. [14] using sequencing grade modified trypsin (EC 3.4.21.4) from Promega for 18 hr at 37°. The eluted peptides were sepd by RPLC on a C18 Vydac (300Å, 5 μm, 2.1 × 150 mm) column at room temp performed with a Spectra-Physics SP 8800 chromatographic device. The gradient– from 0 to 36% MeCN in 80 min with 0.1% CF_3CO_2H in Milli-Q water as buffer A and 0.09% CF_3CO_2H in 60% MeCN– was applied at a flow rate of 0.2 ml min^{-1}. Elution was monitored by *A* at 215 nm and frs were collected manually.

Protein sequencing. For *N*-terminal sequencing of the whole protein, the PVDF membrane with adsorbed AE was cut into 2 × 2 mm pieces, placed in the upper cartridge block of a sequencer equipped with an on-line phenylthiohydantoin amino acid analyser (Applied Biosystems Inc 475 A) . The PVDF pieces were overlayed with a polybrene-coated preconditioned glass fibre disc. The sequencing of the tryptic peptides was conducted with liquid samples from the RPLC sepn. Automated Edman degradation of the whole protein and of peptides

was performed with reagents and methods of the manufacturer.

Homology search in data libraries. A search for sequence homologies was performed using the programme FASTA [15] with the following libraries: NBRF-PIR (release 38), Swissport (release 27) and the translation of the open reading frames of GENBANK (release 80) available at the French data base BISANCE [16]. All computations were done using the similarity matrix of ref. [17].

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