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M_r 33 k OIL BODY ASSOCIATED PROTEIN IN CULTURED SHOOT PRIMORDIA OF *MATRICARIA CHAMOMILLA*

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Key Word Index—*Matricaria chamomilla*; Chamomile; cultured shoot primordia; oil body; oleosin; protease; carboxypeptidase.

Abstract—A major oil body associated protein (p33) was isolated from the cultured shoot primordia of *Matricaria chamomilla*. The M_r of p33 was estimated to be 33 k by gel chromatography and SDS-PAGE. P33 has protease activity which liberates the carboxyl terminal amino acid residues from angiotensin I.

INTRODUCTION

Most plant seeds store oils (triacylglycerols) which are synthesized during seed maturation and mobilized during postgerminative seedling growth [1–5]. The storage oils are confined to subcellular organelles called oil bodies (lipid bodies, oleosomes and spherosomes) [6–8]. The oil bodies have a very simple structure [9, 10]: a central core of triacylglycerols surrounded by a monolayer of phospholipids containing a few proteins [11–16]. However, the function of the protein in oil bodies is not well understood.

Oil bodies were found to propagate remarkably in the cultured shoot primordia of *Matricaria chamomilla* (German chamomile) by addition of auxin [17]. The fact that oil bodies exist even in the multiplying cells (vegetative tissues), which do not need to accumulate substances, led us to investigate another function of oil bodies besides that of storage. To elucidate the physiological function of oil bodies, an oil-body-associated protein was prepared from the oil bodies in the cultured shoot primordia of *Matricaria chamomilla* and its properties were examined.

RESULTS AND DISCUSSION

Oil bodies were isolated from the cultured shoot primordia by centrifugation [17]. Repeated washing of the oil pad resulted in enrichment of the oil body. The oil body was washed with acetone four times to give oil-body-associated proteins. Lane 1 in Fig. 1 shows the SDS-PAGE pattern of the oil body proteins. A strong band was found at the 33 k position on the SDS-polyacrylamide gel. The M_r of the major protein was also estimated to be 33 k by HPLC with a TOSOH G-3000SW. This indicates that the 33 k oil-body-associated protein (p33) is a single polypeptide.

For the peptide mapping, p33 prepared from the oil bodies was incubated with lysyl endopeptidase. The proteolytic fragments were analysed on an SDS-PAGE gel (lane 3 in Fig. 1). More than four fragments (31, 18, 15, and 13 k) were released by the hydrolysis. This fragment pattern could be explained by the peptide map shown in Fig. 2; i.e. the peptide fragment of 31 k can be explained as the sum of the peptide fragments of 18 k and 13 k. The *N*-terminal amino acid sequences of both 31 k and 13 k fragments were Gly-Val-Pro-Glu-Ala. Accordingly, the peptide fragment of 13 k was esti-

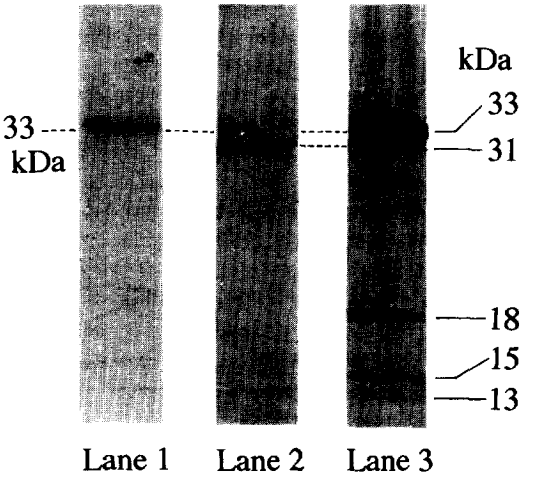

Fig. 1. SDS-PAGE of the oil body protein with or without protease treatment. Lane 1: oil body proteins; Lane 2: proteins after treatment of oil bodies with lysyl endopeptidase; Lane 3: protein after treatment of p33 with lysyl endopeptidase.

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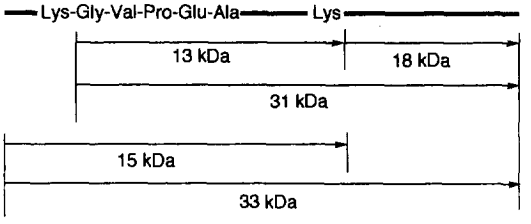

Fig. 2. Peptide map of p33 by lysyl endopeptidase digestion. Positions of lysine which were digested with lysyl endopeptidase are marked with Lys. Peptide fragments shown are those which were revealed on lane-3 of Fig. 1.

mated to be located in middle position of the peptide map.

To clarify the topology of p33 on the oil body, oil bodies themselves were incubated with lysyl endopeptidase and then ruptured by acetone to give a digested protein mixture, which was analysed on SDS-polyacrylamide gel. As shown in lane 2 in Fig. 1, a new band at 31 k was observed. This indicates that a part (2 k fragment) from p33 may be located on the outside of the oil body, because only this part was hydrolysed with lysyl endopeptidase.

Kalinski and his co-workers have reported that the protein associated with soybean seed oil bodies is similar to thiol proteases of the papain family [14]. This report led us to investigate the possibility of p33 as a protease. To clarify the function of p33, protease activity was examined by use of angiotensin I as a substrate, because angiotensin I is a good substrate for both endo- and exoprotease [18, 19]. Angiotensin I was incubated with p33 and then the reaction mixture was subjected to the FAB mass analyses at a regular interval. Signals at *m/z* 1183 [Angiotensin − Leu + H]$^+$, 1046 [Angiotensin − His − Leu + H]$^+$ and 899 [Angiotensin − Phe − His − Leu + H]$^+$ were increased with a lapse of the incubation time. Fig. 3 shows the time-course in the hydrolysis of angiotensin I with p33. This indicates that p33 hydrolysed angiotensin I sequentially from its C-terminal. Leu and His were released rapidly from the carboxyl terminal of angiotensin I, while hydrolysis of the third residue, Phe, from the carboxyl terminal of angiotensin I was relatively slow. A similar observation has been reported on the hydrolysis of some peptides with the carboxypeptidase from citrus [18]. These results show that p33 is a carboxypeptidase, but not an endopeptidase like the protein in the soybean seed oil body [14]. The carboxypeptidase activity became maximally active at pH 6.5, but not active at pH below 5 or above 8.5.

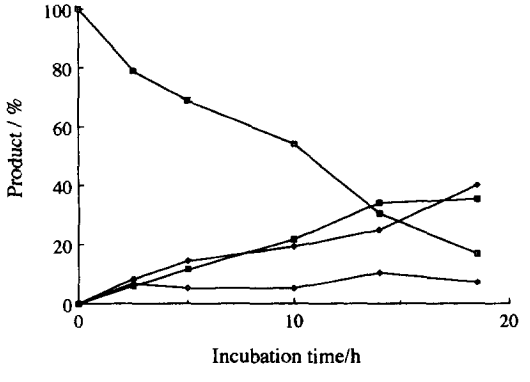

Fig. 3. Time courses in the hydrolysis of angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) with p33. —□— [angiotensin: *m/z* 1296]; —■— [angiotensin − Leu: *m/z* 1189]; —◆— [angiotensin − His-Leu: *m/z* 1046]; —◇— [angiotensin − Phe-His-Leu: *m/z* 899].

Table 1. Effect of inhibitors on carboxypeptidase activity of p33

Reagent	Concentration (mM)	Relative activity* (%)
None	—	100
Iodoacetic acid	0.9	29
PCMB†	0.9	32
E-64§	0.1	20
N-Ethyl maleimide	1.3	83
1,10-Phenanthroline	0.8	93

*Relative activity denotes the ratio of the activity to the control, which was determined under the same conditions except for omission of the inhibitors.

†*p*-Chloromercuribenzoic acid.

§(L-3-trans-Carboxyoxiran-2-carbonyl)-L-leucyl-agmatine.

The effect of some chemical reagents on the carboxypeptidase activity of p33 was examined (Table 1). The activity was inhibited by iodoacetoamide, *p*-chloromercuribenzoic acid (PCMB) and (L-3-trans-carboxyoxiran-2-carbonyl)-L-leucyl agmatine (E-64), but not inhibited by 1,10-phenanthroline and *N*-ethylmaleimide. E-64 and PCMB are known to be specific inhibitors for cysteine protease, and Hg^{2+} in PCMB to react with sulphydryl and carboxyl groups of the protease [20, 21].

Thus it was demonstrated that the oil body protein p33 in the cultured shoot primordia has a carboxypeptidase activity. This protease might act as a cell-protective enzyme, but further work is necessary to clarify the physiological functions of oil body.

EXPERIMENTAL

FAB mass spectra were obtained with JEOL HX-100 double-focusing mass spectrometer fitted with a 18.8 kilogauss magnet and a FAB ion source. The ion source was a 5 kV accelerating potential and FAB was generated by a xenon neutral beam accelerated by a 7 keV potential. The *N*-terminal sequence was determined with an Applied Biosystems 473A automatic sequencer.

Materials. According to the reported procedure [17, 22], shoot primordia of *M. chamomilla* were cultured in test tubes (3 × 20 cm) containing Murashige-Skoog's (MS) liquid media supplemented with 0.02 mg/l^{-1} of α-naphthalene acetic acid (NAA) and 0.2 mg/l^{-1} of 6-benzylaminopurine (BAP) at pH 5.7.

E-64 and angiotensin I (Human) were purchased

from the Peptide Institute (Osaka). Iodoacetoamide, PCMB, 1,10-phenanthroline, *N*-ethylmaleimide and lysyl endopeptidase (Achromobactor protease I; EC 3.4.21.50) were from Wako Pure Chemical Industries.

Isolation of oil body protein. Isolation of oil bodies was performed at 0–4° in all operations. The cultured shoot primordia (50 g) in an equal wt of 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, 0.6 mM $MgCl_2$, and 0.5 M sucrose were homogenized with a mortar for 10 min. The homogenate was filtered through 4 layers of cheesecloth and the filtrate was centrifuged at 10 000 *g* for 30 min to give a floating creamy surface layer. This layer was resuspended in the same buffer and the suspension was centrifuged again at 100 000 *g* for 30 min. The floating oil bodies were collected. This process was repeated more than 3 times until the soln became clear. The oil body fraction was washed with acetone 4 times to remove lipids. The ppt. was then suspended in NaPi buffer (pH 6.5) and stored at 4° overnight to solubilize the protein.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was run in a gel containing 15% (w/v) polyacrylamide and 0.1% (w/v) SDS by the method of ref. [23]. Prior to electrophoresis, the sample protein was heated at 90° for 30 min in 0.01 M Tris-HCl buffer soln (pH 6.8) containing 0.2% (w/v) of SDS, 1% (w/v) of mercaptoethanol and 2% (w/v) of glycerol. The gels were stained with Coomassie Brilliant Blue R. Phosphorylase B (M_r 94 000), bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 30 000), trypsin inhibitor (M_r 20 100) and lysozyme (M_r 14 400) were used as standards.

M_r analysis by HPLC. The M_r of the oil-body-associated protein was estimated from the elution profiles on HPLC with a TOSOH G-3000SW XL column [24]. Thyroglobulin (M_r 670 000), ferritin (M_r 440 000), catalase (M_r 232 000), aldolase (M_r 158 000), bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000), chymotrypsin (M_r 25 000) and RNase (M_r 13 700) were used as standards.

Digestion of oil-body-associated protein in oil body. To a suspension of the oil body (*ca* 4 mg) in Tris-HCl buffer (pH 7.5), lysyl endopeptidase (0.5 nkat) was added and incubated at 37° for 3 hr. After the incubation, the reaction mixture was washed with acetone 4 times to collect the digested oil-body-associated protein. The digested protein mixture was added to 10–15 μl of sample buffer, and SDS-PAGE was run in a gel containing 15% (w/v) polyacrylamide, as described above.

Peptide mapping by limited proteolysis of p33. Peptide mapping by SDS-PAGE was performed with a 0.5 mm-thick slab gel apparatus described in ref. [25]. The sample (7 to 10 μg/10 to 15 μl Tris-HCl buffer (pH 7.5)) was loaded in a sample well and then lysyl endopeptidase (30 mU/10 μl Tris-HCl buffer (pH 7.5)) was overlaid. SDS-PAGE was run as described above. For the *N*-terminal sequence analysis, the SDS gel was electroblotted to a polyvinylidene difluoride (PVDF) membrane at 60 mA according to the reported procedure [26]. The protein bands were visualized on the membrane with Coomassie Brilliant blue and the area of the membrane containing the protein was cut off in ribbons of 2 × 8 mm, each ribbon containing at least 20 μg of protein. Each PVDF ribbon was placed in the upper glass block of the reaction chamber in the protein sequencer and analysed with reversed phase HPLC as phenylthiohydantoin-amino acid derivatives.

Time courses in the hydrolysis of angiotensin I. A 10 mM NaPi buffer soln (pH 6.5; 60 μl) containing angiotensin I (20 nmol) and oil body protein (15 to 30 μg of protein) were incubated at 37° with shaking. At a regular interval, a part (5 to 10 μl) of the incubation mixture was pipetted out and subjected to FAB mass analyses. The FAB MS of angiotensin I gave a mass peak at *m/z* 1296 as a quasi-molecular ion. During digestion, signals at *m/z* 1183 [Angiotensin − Leu + H]$^+$, 1046 [Angiotensin − His − Leu + H]$^+$ and 899 [Angiotensin − Phe − His − Leu + H]$^+$ were observed. The amounts of hydrolysed products and unchanged substrate were determined on the basis of the peak intensity on the mass spectrum.

Effects of inhibitors on carboxypeptidase activity. P33 (15 to 30 μg of protein) in 50 μl of 10 mM NaPi buffer (pH 6.5) containing inhibitors was preincubated at 35° for 10 min. To the mixture, angiotensin I (20 nmol) was added and incubated at 35° for 10 hr. After incubation, the reaction mixture was chilled at 0° and then centrifuged. A part (*ca* 10 μl) of the supernatant was analysed by FAB mass spectrometer. Carboxypeptidase activity was determined as the decrease of the signal intensity at *m/z* 1296, which is assigned as the molecular ion of unchanged angiotensin I.

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